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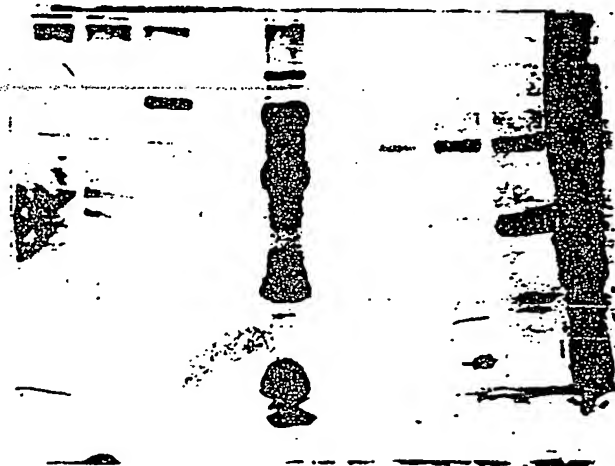
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(57) Abstract

Multifunctional proteins having M-CSF activity and at least one other bioactivity not found together in a single naturally occurring molecule are described. These multifunctional M-CSF proteins can be produced by the expression of fused genes which are also described. These multifunctional M-CSF proteins have increased therapeutic potential.

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MULTIFUNCTIONAL M-CSF PROTEINS AND
GENES ENCODING THEREFOR

The present invention is in the field of biotechnology. More
specifically, it relates to multifunctional proteins having two or more biological

5   activities that are not found together in a single naturally occurring molecule. It further
relates to the recombinant DNA that codes for M-CSF fusion proteins, the recombinant
vectors that include the DNA, host organisms transformed with the recombinant vectors
that produce the proteins, methods for producing the fusion proteins, pharmaceutical
compositions containing the proteins and therapeutic methods employing the proteins.

10   Recent technology has permitted the design of hybrid molecules which
do not naturally occur. Fused selectable markers known as fusion flags to facilitate
cloning, secretory leader peptide fusions to produce extracellular products and
immunoconjugates are examples of such hybrid molecules. There have also been
reports of attempts to produce hybrid proteins which possess potentially novel

15   therapeutic properties by virtue of the combination of functions derived from each of
the parent molecules. For example, hybrid interferons (IFNs) are disclosed in U.S.
Patent No. 4,758,428 and in European Patent Publication No. 0225579. Feng, G.-S.
et al. (1988, Science 241:1501-1503) discloses a hybrid protein between IFN-γ and
TNF-β which has increased antiproliferative activity in vitro compared with either IFN-

20   γ or TNF-β alone. Japanese Patent Publication No. 61128889 discloses fusion
proteins between IFN and urokinase. Hybrid plasminogen activators comprising, for
example, fusions of urokinase and tissue plasminogen activator have also been
reported. There are numerous disclosures of polypeptide-toxin hybrid proteins
notwithstanding immunotoxins. For example, Kelley, V.E. et al. (1988, Proc. Natl.

25   Acad. Sci. USA 85:3980-3984) report an IL-2/diphtheria toxin fusion protein which
was found to be a potent immunosuppressive agent. U.S. Patent No. 4,545,985
discloses IL-2/Pseudomonas exotoxin fusion proteins.

Another IL-2 fusion protein is disclosed in Australian Patent Abstract
AU-A-66756/86 and European Patent Publication No. 0288809 (corresponding to PCT

30   Patent Publication No. WO 87/02060) which report fusion proteins consisting of IL-2
and GM-CSF. The IL-2 sequence can be at either the N- or C-terminal end of the GM-
CSF such that after acid cleavage of the fusion protein, GM-CSF having either N- or C-
terminal sequence modifications can be generated.

In contrast, the present invention provides for the production of

35   multifunctional proteins comprising M-CSF bioactivity produced by chemical or genetic

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fu ion. Thus, the invention provides for the production of dimeric fusion proteins. It is urprising that these complex multifunctional homodimeric and heterodimeric proteins can be produced having two or more bioactivities since the refolding of M-CSF protein alone into its bioactive form is a complicated process. Furthermore, the
5   multifunctional fusion proteins of the invention have increased therapeutic potential due to their multifunctional nature and their increased circulating half-life.

The present invention relates to DNA sequences encoding novel multifunctional proteins having two or more bioactivities not found together in a single naturally occurring molecule. The present invention further relates to a DNA sequence
10   encoding a multifunctional fusion protein wherein one function of said protein in it active form is to stimulate the formation of primarily macrophage colonies in the in vitro colony stimulating assay of Ralph, P. et al., 1986, Blood 68:633. Since, M-CSF is active in its dimeric form, the invention relates to DNA sequences encoding multifunctional fusion proteins that are dimers. Further, the invention relates to DNA
15   sequences encoding fusion proteins having M-CSF bioactivity and a second bioactivity associated with a protein selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, α-IFN, β-IFN, γ-IFN, G-CSF, GM-CSF, TNF-α, TNF-ß, erythropoietin (EPO), thrombopoietin or other platelet enhancing factors, ricin A, diphtheria toxin and Pseudomonas exotoxin. The invention also relates to DNA
20   sequences having the M-CSF bioactivity i.e., the function of stimulating the formation of primarily macrophage colonies in the in vitro assay, encoded 5' to the second bioactivity. Among other surprising results, the present invention provides DNA sequences that encode for M-CSF/IL-2 fusion monomers that associate into dimers having both M-CSF and IL-2 bioactivities and that have the potential for a longer half-
25   life due to the larger molecular size of the fused molecule.

Furthermore, the present invention relates to DNA sequences encoding for a multifunctional fusion protein including an amino acid sequence substantially equivalent to the amino acid sequence of M-CSF. The invention further relates to DNA sequences encoding the amino acid sequence of M-CSF which additionally encode the
30   amino acid sequence substantially equivalent to the amino acid sequence of IL-2, IL-1α, IL-1ß, IFN-γ, G-CSF or IL-6. The invention also relates to DNA sequences comprising coding sequence from a second protein in addition to M-CSF which hybridize under stringent conditions to the DNA encoding an amino acid sequence substantially equivalent to that of M-CSF or its complementary strand. DNA sequences
35   comprising coding sequence from IL-2, IL-1α, IL-1ß, IFN-γ, G-CSF or IL-6 in addition to M-CSF which hybridize under stringent conditions to the fused genes are embodiments of the present invention as well. The cell cultures transformed with the

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DNAs described and the expression systems comprising the DNAs described are also
among the embodiments of the present invention.

Additionally, the present invention relates to novel multifunctional
proteins wherein one function of the protein in its active form is to stimulate the
5   formation of primarily macrophage colonies in the in vitro colony stimulating assay of
Ralph, P. et al., (supra). The invention further relates to multifunctional proteins
which are fusion proteins and which are dimers. Additionally, the invention relates to
multifunctional proteins comprising M-CSF activity and a second bioactivity associated
with a protein selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6,
10 IL-7, α–IFN, β-IFN, γ-IFN, G-CSF, GM-CSF, TNF-α, TNF-ß, EPO,
thrombopoietin or other platelet enhancing factors, ricin A, diptheria toxin and
Pseudomonas exotoxin. The invention further relates to multifunctional proteins
wherein the function of stimulating the formation of primarily macrophage colonies in
the in vitro assay resides in the amino-terminal end of the molecule. Also included in
15 the invention are multifunctional proteins whch are heterodimers comprising
monomeric subunits each having different second bioactivity associated with a protein
selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, α–
IFN, β-IFN, γ-IFN, G-CSF, GM-CSF, TNF-α, TNF-ß, EPO, thrombopoietin or
other platelet enhancing factors, ricin A, diptheria toxin and Pseudomonas exotoxin.
20   Figure 1 shows immunoaffinity purified multifunctional fusion products
encoded by pML1 separated by SDS-PAGE and stained with Coomassie blue.
As used herein, the term "multifunctional M-CSF proteins" refers to
proteins having the ability to stimulate the formation of primarily macrophage colonies
in the in vitro colony stimulating assay of Ralph, P. et al., supra and at least one other
25 biological activity which are conjugated either chemically or genetically and which are
not found together in a single naturally occurring molecule. Hybrid genes which
encode for these multifunctional M-CSF fusion proteins are produced by recombinant
DNA technology. The multifunctional fusion proteins disclosed herein have
macrophage colony stimulating factor activity as described below as one of their
30 bioactivities. This M-CSF bioactivity may result from the M-CSF coding sequence at
either the amino-terminal or carboxy-terminal end of the hybrid gene. Additionally,
since native M-CSF is a dimer, the multifunctional fusion proteins of the invention as
disclosed herein occur as dimeric or higher multimeric fusion proteins. Each
monomeric subunit of these dimeric fusion proteins may have a second bioactivity. In
35 the case of heterodimeric fusion proteins, only one subunit may have a second
bioactivity or, in the alternative, each subunit may have a different second bioactivity
resulting, in fact, in a trifunctional heterodimeric M-CSF fusion protein.

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In addition to the combination of activities genetically, it should be clear
that the hybrid proteins disclosed herein can be made by chemically conjugating
portions of the M-CSF protein with the second bioactive protein. Examples of
chemical conjugation are shown in U.S. Patent Nos. 4,468,382 and 4,545,985, in

5   Pinker et al., 1985, J. Clin. Invest. 76:1261-1267, Veronese, et al., 1985, Biochem. &
Biotech. 11:141-152 and in commonly owned U.S. Serial No. 146,275 each of which
is incorporated herein by reference in its entirety. Since native M-CSF is a dimer, the
conjugated multifunctional proteins of the invention can occur as dimeric or higher
multimeric proteins. Each M-CSF monomer of the multifunctional proteins may be

10 conjugated to a second bioactivity. In the case of heterodimeric conjugates, chemical
coupling is carried out with monomeric M-CSF followed by refolding a mixture of
differentially conjugated M-CSF monomers into active forms. Alternatively, by using
an intact (refolded) M-CSF protein which has different monomeric subunits, each
monomer can be selectively conjugated to different second bioactive proteins. Only one

15 M-CSF subunit may have a second bioactivity or, in the alternative, each subunit may
have a different second bioactivity resulting, in fact, in a trifunctional heterodimeric M-
CSF protein. The M-CSF conjugates envisioned may have a more complex multimeric
structure dependent upon the nature of the protein having the second bioactivity, eg.
antibodies.

20   M-CSF can be coupled to toxin molecules including diphtheria toxin,
ricin A toxin or Pseudomonas exotoxin from which the cell-recognition portion of the
toxin molecule has been removed. Such multifunctional CSF-toxin conjugates are
useful in the treatment of myeloid leukemia, autoimmune diseases, inflammation, graft
rejection or other diseases when the target cells express M-CSF receptors. M-CSF can

25 also be fused to blood proteins which target bone marrow blood cell precursors
including IL-1, IL-3, IL-6, GM-CSF and G-CSF. M-CSF can be coupled to
antibodies that result in similar localization to bone marrow blood cell precursors.
Preferably the proteins appropriate for the multifunctional proteins described herein
include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, α-IFN, β-IFN, γ-IFN, G-CSF,

30 GM-CSF, TNF-α, TNF-β, EPO, thrombopoietin or other platelet enhancing factors,
ricin A, diptheria toxin and Pseudomonas exotoxin. EPO, thrombopoietin or other
platelet enhancing factors, ricin A, diphtheria toxin and Pseudomonas exotoxin. Most
preferably the proteins appropriate for the fusions described herein are M-CSF, IL-2,
IL-3, IL-6, G-CSF and IFN-γ. The interactions of some of these factors has been

35 recently reviewed (Ralph, P. et al., in The Year In Immunology 1988, Cruse et al.,
eds., Basel, Karger, 1989, Vol. 5, pp. 103-125; Ralph, P., "Colony Stimulating

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Fac rs" in Human Monocytes Asherson et al., eds. Academic Press, 1989, pp. 227-246.

As used herein, the term "M-CSF" refers to recombinant macrophage colony stimulating factor or macrophage colony stimulating factor-like proteins

5   produced by a transformed host cell whose amino acid sequence is the same as, similar to, or substantially equivalent to the unglycosylated and/or glycosylated native macrophage colony stimulating factor. Substantially equivalent means the sequences are identical or differ by one or more amino acid alterations (deletions, additions or substitutions) that do not cause an adverse functional dissimilarity in biological activity

10   between the synthetic and native protein. M-CSF is a protein which exhibits the spectrum of activity understood in the art for M-CSF--also known as CSF-1 i.e., when applied to the standard in vitro colony stimulating assay of Metcalf, D., J. Cell. Physiol. (1970) 76:89 as modified by Ralph, P. et al., supra, it is capable of stimulating the formation of primarily macrophage colonies. Native M-CSF is a

15   glycosylated dimer; dimerization is reported to be necessary for activity as the monomer is not active in the Metcalf or Ralph colony stimulating assays (supra) or various other in vitro bioactivity assays (Das, S.K. et al., 1981, Blood 58:630-641; Das, S.K. et al., 1982, J. Biol. Chem. 257:13679-13681; Stanley, E.R. et al., 1977, J. Biol. Chem. 252:4305-4312, Halenbeck, R. et al., 1989, Bio/Technology in press).

20   It should be noted that a low level of activity was observed in the assay of a monomer used by Halenbeck et al. supra but this was due to dimerization of M-CSF during the assay. The monomeric form may be converted to the dimeric form by in vitro provision of suitable refolding conditions, and the monomer is per se useful as an antigen to produce anti-M-CSF antibodies.

25   There appears to be some species specificity: Human M-CSF is operative both on human and on murine bone marrow cells; murine M-CSF does not show activity with human cells. Therefore, "human" M-CSF should be positive in the specific murine radioreceptor assay of Das et al., 1981, (supra), although there is not necessarily a complete correlation. The biological activity of the M-CSF protein will

30   generally also be inhibited by neutralizing antiserum to human urinary M-CSF (Das, S.K. et al., 1981, supra). However, in certain special circumstances this criterion may not be met. For example, a particular antibody preparation may recognize a M-CSF epitope not essential for biological function, and which epitope is not present in the particular M-CSF mutein being tested or which is obscured by the tertiary structure of

35   the M-CSF fusion protein. For purposes of definition herein, the M-CSF must (1) stimulate the formation of monocyte-macrophage colonies using bone marrow cells from the appropriate species as starting materials, (2) under most circumstances (see

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ab... e) show inhibition of this activity by neutralizing antiserum against purified human
uri...ary M-CSF, and(3) where appropriate for species type, exhibit a positive response
to ... e radioreceptor assay.

5 M-CSF apparently occurs in numerous forms all which are included in
the embodiments of the present invention. Human M-CSF cDNA clones coding for M-
CS: proteins of three different lengths (α, 256 amino acids; β, 554 amino acids; and γ,
43? amino acids) have been isolated from cells expressing the single M-CSF gene
(Wong, et al., 1987, Science 235:1504-1508; Kawasaki, et al., 1985, Science 230:291-
29?: Ladner et al., 1987, Embo J. 6:2693-2698; Cerretti, D.P. et al., 1988, Molecular
10 Immunol. 25:761-770). The M-CSF proteins useful in the multifunctional proteins
disclosed herein may also be processed by proteolysis. It is believed that M-CSF may
occur in nature in one or more C-terminally deleted forms. In addition, M-CSFs
lacking the first two or four amino acids have been isolated in active form from the
supernatant of the human cell line AGR-ON (equivalent to CEM-ON; ATCC No. CRL-
15 8199; Takahashi, M. et al., 1988, Bioch. Biophys. Res. Comm. 152:1401-1409). M-
CSF protein comprising monomers ending at amino acid 145 are reported to have in
vitro biological activity (European Patent Publication No. 0261592 published March
30, 1988 incorporated herein by reference in its entirety). The monomeric M-CSF
polypeptide (whether clipped at the C-terminus or not) may also refold to form
20 multimers, most frequently dimers.

Thus, a protein including an amino acid sequence substantially
equivalent to the amino acid sequence of M-CSF comprising: Glu-Tyr-Cys-Ser-His-
Met-Ile-Gly-Ser-Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-
Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-Lys-Asp-Pro-Val-
25 Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-Asp-Ile-Met-Glu-Asp-Thr-Met-Arg-
Phe-Arg-Asp-Asn-Thr-Pro-Asn-Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-
Arg-Leu-Lys-Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-Arg-
Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-Asn-Val-Phe-Asn-Glu-
Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-
30 Phe-Ala-Glu and the DNA sequence encoding therefor are considered to be within the
scope of the instant invention.

Native human urinary M-CSF has been isolated as a highly
glycosylated dimer of 45-90 kd, depending on the source, method of measurement and
identity of the reporter. The recombinantly produced unglycosylated M-CSF reported
35 by Wong, G.G. et al. supra, appears to have a subunit molecular weight of
approximately 21 kd. On the other hand, the molecular weight calculated on the basis
of the amino acid sequence deduced for the "short" 224 amino acid form of CSF

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(M-CSF) by Kawasaki, E.S. et al. (supra) (also U.S. Patent No. 4,847,201 issued July 11 1989 and PCT Patent Publication No. WO 86/04607 published August 14, 1986 which is incorporated herein by reference in its entirety) is on the order of 26 kd, while the of the "long" 522 amino acid form (LCSF) is calculated to be on the order to 55 kd

5 (Wong, G.G. et al. (supra); Ladner, M.B. et al.(supra); commonly owned European Patent Publication No. 0272779 published June 29, 1988; and PCT Patent Publication No. WO 87/06954 published November 19, 1987 each of which is incorporated herein by reference in its entirety). When deleted constructs of these genes are expressed in E. coli (where glycosylation does not occur), they, of course, give rise to proteins of

10 considerably lower molecular weight.

It is, of course, well known that bacterially produced mature proteins which are immediately preceded by an ATG start codon may or may not include the N-terminal methionine in the form as produced and recovered. In addition, slight modification of the N-terminal sequence may aid in the processing of the N-terminal

15 methionine, and it is shown in commonly owned European Publication No. 0272779, that deletion of residues 1 and 2 (both glutamic acid) or residues 1-3 (glu-glu-val) aids in this manner. Deletions are noted by a ∇ followed by the number of amino acids deleted from the N-terminal sequence, or by the number of amino acids remaining when residues are deleted from the C-terminal sequence. Thus, the N-terminal

20 deletions referred to above having the first 2 and the first 3 residues deleted are designated N ∇ 2 and N ∇ 3, respectively. C-terminal truncations of M-CSF resulting in proteins of 150, 158, 190 or 221 amino acids in length for example are referred to as C ∇150, C∇158, C∇190 and C∇ 221, respectively. A 221 amino acid M-CSF molecule derived from the long form LCSF having an N-terminal deletion of 3 amino

25 acids is denoted by LCSF/N∇ 3 C∇221 for example. Amino acid substitutions are designated by reference to the position of the amino acid which is replaced. For example, substitution of the cysteine residue at position 157 in Figure 4 of Ladner et al. (supra) by serine is referred to as M-CSF ser$_{157}$. Accordingly, all of these forms may be used in the fusion proteins produced by the process disclosed herein.

30 In summary, in addition to the N-terminal and C-terminal deletions and aggregations, individual amino acid residues in the chain may be modified by oxidation, reduction, deletion or other derivatization, and these proteins may also be cleaved and/or polymerized to obtain products that retain activity. Such alterations which do not destroy activity do not remove the protein sequence from the definition,

35 and are specifically included as substantial equivalents. M-CSF derived from other species may fit the definition of a protein having activity of "human M-CSF" by virtue

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o. its display of the requisite pattern of activity as set forth above with regard to human substrate.

As used herein, the term "IL-2" refers to recombinant interleukin-2 or interleukin-2-like proteins produced by a transformed host cell and whose amino acid
5   sequence is the same as or similar or substantially equivalent to the unglycosylated and/or glycosylated native human interleukin-2. Examples of such IL-2s are those described in European Patent Publication Nos. 091,539, 088,195, 109,748 and 200,280; those described in commonly owned U.S. Patent Nos. 4,518,584 and 4,752,585 and in U.S. Patent Nos. 4,738,927 and 4,778,879, each of which is
10   incorporated herein by reference in its entirety; IL-2 described by Taniguchi et al., 1983, Nature 302:305-310 and Devos et al., 1983, Nucleic Acids Res. 11:4307-4323; and bovine IL-2 as described by Cerretti et al., 1986, Proc. Natl. Acad. Sci USA 83:3223-3227. The disclosures of all these references are incorporated herein by reference.

15   The IL-2s preferred herein include biologically active muteins (analogs) of human IL-2 in which amino acid residues not essential to biologically activity have been deliberately deleted or replaced with a conservative amino acid as indicated below. The gene encoding des-alanyl-IL-2$_{cys.125}$ is available in pLW1 deposited as ATCC No. 39405. IL-2s preferred in the multifunctional proteins of the invention also include
20   those wherein the cysteine residue at position 125 is replaced with another amino acid, preferably neutral or conservative, to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide and, optionally, the N-terminal alanine residue of the native counterpart is eliminated. By a "conservative" amino acid substitution is meant one which does not change the activity characteristics of the protein, and in general is
25   characterized by chemical similarity of the side chains of the two residues interchanged. for example, acidic residues are conservatively replaced by other acidic residues, basic by basic, hydrophobic by hydrophobic, bulky by bulky, and so forth. The degree of similarity required depends, of course, on the criticality of the amino acid for which substitution is made, and its nature. Thus, in general, preferred substitutions for
30   cysteine residues are serine and alanine; for aspartic acid residues, glutamic acid; for lysine or arginine residues, histidine; for leucine residues, isoleucine, or valine; for tryptophan residues, phenylalanine or tyrosine; and so forth. More particularly, IL-2 muteins useful in the multifunctional proteins of this invention are those wherein (1) the cysteine residue at amino acid position 125 of the native counterpart is replaced by a
35   serine residue (designated IL-2$_{ser125}$; encoded in pLW55 deposited as ATCC No. 39516) or alanine residue (designated IL-2$_{ala125}$); or (2) the initial alanine residue is

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eliminated and the cysteine at position 125 is replaced by serine (designated des-alanyl-IL-2$_{ser125}$; encoded in pLW45 deposited as ATCC No. 39626).

Other IL-2s useful herein are those biologically active muteins described in U.S. Patent No. 4,752,585 issued June 21, 1988 (equivalent to European Patent
5   Publication No. 200,280 published December 10, 1986) wherein oxidation-susceptible methionine residues are replaced with a neutral or conservative amino acid, a preferred mutein includes replacing the methionine at position 104 with a conservative amino acid such as alanine.

EPO 200,280 also describes amino-terminal deletions of IL-2 wherein
10 one or more of the first six amino acids are deleted. Other amino-terminal deletions of IL-2 are disclosed in Chemical Abstracts, 1987, 106:(21):170236f, an abstract of Japanese Patent Publication No. 61/225,199 published October 1986, wherein any one of the first 15 amino acids of IL-2 are deleted. PCT Patent Publication No. WO 87/04714, published August 13, 1987 describes deletions or replacements of one or
15 more of the amino acid residues in positions 2 to 11 and/or 128 to 133 from the amino-terminal alanine of IL-2. Thus, a multifunctional M-CSF protein including an amino acid sequence substantially equivalent to the amino acid sequence of IL-2 comprising: Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-Leu-Leu-Asp-Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-
20 Tyr-Met-Pro-Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Glu-Glu-Leu-Lys-Pro-Leu-Glu-Glu-Val-Leu-Asn-Leu-Ala-Gln-Ser-Lys-Asn-Phe-His-Leu-Arg-Pro-Arg-Asp-Leu-Ile-Ser-Asn-Ile-Asn-Val-Ile-Val-Leu-Glu-Leu-Lys-Gly-Ser-Glu-Thr-Thr-Phe-Met-Cys-Glu-Tyr-Ala-Asp-Glu-Thr-Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser-Ile-Ile-Ser-Thr-Leu-Thr and DNA sequences encoding
25 therefor are considered to be within the scope of the instant invention.

As used herein the term "IFN-γ" refers to recombinant interferon-gamma or interferon-gamma-like proteins produced by a transformed host cell and whose amino acid sequence is the same as or substantially equivalent to the unglycosylated and/or glycosylated native human or murine interferon-gamma. The
30 IFN-γs preferred herein are those biologically active, essentially full-length forms, of IFN-γ. Human IFN-γ and derivatives thereof have been described in U.S. Patent No. 4,762,791 issued August 9, 1988 and incorporated herein by reference in its entirety. Human IFN-γ beginning with the 6th amino acid, Pro, and ending at the 127th amino acid, Ala, are reported to be active (European Patent Publication No.
35 219,781, published April 29, 1987 and incorporated herein by reference in its entirety). Thus, a multifunctional M-CSF protein including an amino acid sequence substantially equivalent to the amino acid sequence of human IFN-γ comprising: Pro-Tyr-Val-Lys-

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Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Ala-Gly-His-Ser-Asp-Val-Ala-Asp-Asn-
Gly-Thr-Leu-Phe-Leu-Gly-Ile-Leu-Lys-Asn-Trp-Lys-Glu-Glu-Ser-Asp-Arg-Lys-Ile-
Met-Gln-Ser-Gln-Ile-Val-Ser-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-Lys-Asp-Asp-
Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-Asp-Met-Asn-Val-Lys-Phe-Phe-
5   Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-Lys-Leu-Thr-Asn-Tyr-Ser-Val-Thr-
Asp-Leu-Asn-Val-Gln-Arg-Lys-Ala-Ile-His-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-
Ser-Pro-Ala-Ala and DNA sequences encoding therefor are considered to be within the
scope of the instant invention.

Murine IFN-γ and derivatives thereof have been described in Gray,
10   P.W. et al., (1983, Proc. Natl. Acad. Sci. USA 80:5842-5846) and are used herein
because IFN-γ is species specific. This is, human IFN-γ is not active in an assay
using a murine cell line. Proteins having these amino acid sequences or substantially
equivalent sequences which result in a proteins having the bioactivity understood in the
art for IFN-γ are considered to be within the scope of the invention. Thus, a
15   multifunctional M-CSF protein including an amino acid sequence substantially
equivalent to the amino acid sequence of murine IFN-γ comprising: Cys-Tyr-Cys-His-
Gly-Thr-Val-Ile-Glu-Ser-Leu-Glu-Ser-Leu-Asn-Asn-Tyr-Phe-Asn-Ser-Ser-Gly-Ile-
Asp-Val-Glu-Glu-Lys-Ser-Leu-Phe-Leu-Asp-Ile-Trp-Arg-Asn-Trp-Gln-Lys-Asp-Gly-
Asp-Met-Lys-Ile-Leu-Gln-Ser-Gln-Ile-Ile-Ser-Phe-Tyr-Leu-Arg-Leu-Phe-Glu-Val-Leu-
20   Lys-Asp-Asn-Gln-Ala-Ile-Ser-Asn-Asn-Ile-Ser-Val-Ile-Glu-Ser-His-Leu-Ile-Thr-Thr-
Phe-Phe-Ser-Asn-Ser-Lys-Ala-Lys-Lys-Asp-Ala-Phe-Met-Ser-Ile-Ala-Lys-Phe-Glu-
Val-Asn-Asn-Pro-Gln-Val-Gln-Arg-Gln-Ala-Phe-Asn-Glu-Leu-Ile-Arg-Val-Val-His-
Gln-Leu-Leu-Pro-Glu-Ser-Ser-Leu-Arg-Lys-Arg-Lys-Arg-Ser-Arg-Cys and DNA
sequences encoding therefor are considered to be within the scope of the instant
25   invention.

"G-CSF" as used herein refers to a recombinant protein having the
effect of stimulating the production of primarily neutrophil colonies or neutrophil-
macrophage colonies in a colony forming assay using bone marrow cell progenitors of
an appropriate species. A protein having this activity and whose amino acid sequence is
30   the same as or similar or substantially equivalent to unglycosylated and/or glycosylated
native human G-CSF as disclosed in European Patent Publication No. 256,843,
published February 24, 1988 or U.S. Patent No. 4,810,643 issued March 7, 1989,
incorporated herein by reference in its entirety, is considered to be within the scope of
the invention. Thus, a multifunctional M-CSF protein including an amino acid
35   sequence substantially equivalent to the amino acid sequence of G-CSF comprising:
Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-
Val-Arg-Lys-Ile-Gln-Gly-Asp-Gly-Ala-Ala-Leu-Gln-Glu-Lys-Leu-Cys-Ala-Thr-Tyr-

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Lys-Leu-Cys-His-Pro-Glu-Glu-Leu-Val-Leu-Leu-Gly-His-Ser-Leu-Gly-Ile-Pro-Trp-
Ala-Pro-Leu-Ser-Ser-Cys-Pro-Ser-Gln-Ala-Leu-Gln-Leu-Ala-Gly-Cys-Leu-Ser-Gln-
Leu-His-Ser-Gly-Leu-Phe-Leu-Tyr-Gln-Gly-Leu-Leu-Gln-Ala-Leu-Glu-Gly-Ile-Ser-
Pro-Glu-Leu-Gly-Pro-Thr-Leu-Asp-Thr-Leu-Gln-Leu-Asp-Val-Ala-Asp-Phe-Ala-Thr-
5   Thr-Ile-Trp-Gln-Gln-Met-Glu-Glu-Leu-Gly-Met-Ala-Pro-Ala-Leu-Gln-Pro-Thr-Gln-
Gly-Ala-Met-Pro-Ala-Phe-Ala-Ser-Ala-Phe-Gln-Arg-Arg-Ala-Gly-Gly-Val-Leu-Val-
Ala-Ser-His-Leu-Gln-Ser-Phe-Leu-Glu-Val-Ser-Tyr-Arg-Val-Leu-Arg-His-Leu-Ala-
Gln-Pro and DNA sequences encoding therefor are considered to be within the scope of
the instant invention.

10   As used herein, the term "IL-1" refers to recombinant interleukin-1 or
interleukin-1-like proteins produced by a transformed host cell and whose amino acid
sequence is the same as or substantially equivalent to the unglycosylated and/or
glycosylated native human interleukin-1. Human interleukin-1 has been described in
U.S. Patent No. 4,801,686 issued January 31, 1989, in European Patent Publication
15   No. 165654 published December 27, 1985, in PCT Patent Publication No. WO
85/00830 published February 28, 1985, and in European Patent Publication No.
267629 published May 18, 1988 each incorporated herein by reference in its entirety.
DNAs encoding for IL-1 have been described in European Patent Publication Nos.
188864 published July 30, 1986 and 200986 published November 12, 1986, European
20   Patent Publication No. 259160 published March 9, 1988 each incorporated herein by
reference in its entirety. In addition, two forms of the IL-1 polypeptide, referred to as
IL-1alpha (IL-1α) and IL-1beta (IL-1β), have been described (human IL-1α in March,
C.J. et al., 1985, Nature 315:641 and human IL-1β in Auron, P.E. et al., 1984, Proc.
Natl. Acad. Sci. USA 81:7907)). Both forms are considered in the definition of IL-1
25   as used herein. Thus, a multifunctional M-CSF protein including an amino acid
sequence substantially equivalent to the amino acid sequence of IL-1α comprising: Met-
Arg-Ile-Ile-Lys-Tyr-Glu-Phe-Ile-Leu-Asn-Asp-Ala-Leu-Asn-Gln-Ser-Ile-Ile-Arg-Ala-
Asn-Asp-Gln-Tyr-Leu-Thr-Ala-Ala-Ala-Leu-His-Asn-Leu-Asp-Glu-Ala-Val-Lys-Phe-
Asp-Met-Gly-Ala-Tyr-Lys-Ser-Ser-Lys-Asp-Asp-Ala-Lys-Ile-Thr-Val-Ile-Leu-Arg-Ile-
30   Ser-Lys-Thr-Gln-Leu-Tyr-Val-Thr-Ala-Gln-Asp-Glu-Asp-Gln-Pro-Val-Leu-Leu-Lys-
Glu-Met-Pro-Glu-Ile-Pro-Lys-Thr-Ile-Thr-Gly-Ser-Glu-Thr-Asn-Leu-Leu-Phe-Phe-
Trp-Glu-Thr-His-Gly-Thr-Lys-Asn-Tyr-Phe-Thr-Ser-Val-Ala-His-Pro-Asn-Leu-Phe-
Ile-Ala-Thr-Lys-Gln-Asp-Tyr-Trp-Val-Cys-Leu-Ala-Gly-Gly-Pro-Pro-Ser-Ile-Thr-Asp-
Phe-Gln-Ile-Leu and DNA sequences encoding therefor are considered to be within the
35   scope of the instant invention.

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Similarly, a multifunctional M-CSF protein including an amino acid
sequence substantially equivalent to the amino acid sequence of IL-1ß comprising: Arg-
Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-Ser-Gln-Gln-Lys-Ser-Leu-Val-Met-Ser-Gly-Pro-
Tyr-Glu-Leu-Lys-Ala-Leu-His-Leu-Gln-Gly-Gln-Asp-Met-Glu-Gln-Gln-Val-Val-Phe-
5   Ser-Met-Ser-Phe-Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys-Ile-Pro-Val-Ala-Leu-Gly-
Leu-Lys-Glu-Lys-Asn-Leu-Tyr-Leu-Ser-Cys-Val-Leu-Lys-Asp-Asp-Lys-Pro-Thr-Leu-
Gln-Leu-Glu-Ser-Val-Asp-Pro-Lys-Asn-Tyr-Pro-Lys-Lys-Lys-Met-Glu-Lys-Arg-Phe-
Val-Phe-Asn-Lys-Ile-Glu-Ile-Asn-Asn-Lys-Leu-Glu-Phe-Glu-Ser-Ala-Gln-Phe-Pro-
Asn-Trp-Tyr-Ile-Ser-Thr-Ser-Gln-Ala-Glu-Asn-Met-Pro-Val-Phe-Leu-Gly-Gly-Thr-
10   Lys-Gly-Gly-Gln-Asp-Ile-Thr-Asp-Phe-Thr-Met-Gln-Phe and DNA sequences
encoding therefor are considered to be within the scope of the instant invention.

As used herein, the term "IL-6" refers to recombinant interleukin-6 or
interleukin-6-like proteins produced by a transformed host cell and whose amino acid
sequence is the same as or substantially equivalent to the unglycosylated and/or
15   glycosylated native human interleukin-6. Human interleukin-6, also referred to as
human B-cell differentiation factor, has been described by Hirano, T. et al.(1986,
Nature 324:73-76, incorporated herein by reference in its entirety). Like IL-1, IL-6
affects very primitive hemopoietic cells and its multiple actions have been discussed in
Wong, G.G., et al. (1988, Immunol. Today 9:137). Thus, a multifunctional M-CSF
20   protein including an amino acid sequence substantially equivalent to the amino acid
sequence of IL-6 comprising: Pro-Val-Pro-Pro-Gly-Glu-Asp-Ser-Lys-Asp-Val-Ala-
Ala-Pro-His-Arg-Gln-Pro-Leu-Thr-Ser-Ser-Glu-Arg-Ile-Asp-Lys-Gln-Ile-Arg-Tyr-Ile-
Leu-Asp-Gly-Ile-Ser-Ala-Leu-Arg-Lys-Glu-Thr-Cys-Asn-Lys-Ser-Asn-Met-Cys-Glu-
Ser-Ser-Lys-Glu-Ala-Leu-Ala-Glu-Asn-Asn-Leu-Asn-Leu-Pro-Lys-Met-Ala-Glu-Lys-
25   Asp-Gly-Cys-Phe-Gln-Ser-Gly-Phe-Asn-Glu-Glu-Thr-Cys-Leu-Val-Lys-Ile-Ile-Thr-
Gly-Leu-Leu-Glu-Phe-Glu-Val-Tyr-Leu-Glu-Try-Leu-Gln-Asn-Arg-Phe-Glu-Ser-Ser-
Glu-Glu-Gln-Ala-Arg-Ala-Val-Gln-Met-Ser-Thr-Lys-Val-Leu-Ile-Gln-Phe-Leu-Gln-
Lys-Lys-Ala-Lys-Asn-Leu-Asp-Ala-Ile-Thr-Thr-Pro-Asp-Pro-Thr-Thr-Asn-Ala-Ser-
Leu-Leu-Thr-Lys-Leu-Gln-Ala-Gln-Asn-Gln-Trp-Leu-Gln-Asp-Met-Thr-Thr-His-Leu-
30   Ile-Leu-Arg-Ser-Phe-Lys-Glu-Phe-Leu-Gln-Ser-Ser-Leu-Arg-Ala-Leu-Arg-Gln-Met
and DNA sequences encoding therefor are considered to be within the scope of the
instant invention.

The precise chemical structure of the M-CSF, G-CSF, IL-2, IFN-γ IL-
6 or IL-1 protein depends on a number of factors. As ionizable amino and carboxyl
35   groups are present in the molecule, a particular protein may be obtained as an acidic or
basic salt, or in neutral form. All such preparations which retain their activity when
placed in suitable environmental conditions are included in the definition of proteins

herein. Further, the primary amino acid sequence of the protein may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like, as well as by conjugation with saccharides, polyethylene glycols (PEGs) and polyoxyethylene glycols (POGs).

5   Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced in vitro. In any event, such modifications are included in the definition of protein herein so long as the activity of the protein, as defined above, is not destroyed. It is expected, of course, that such modifications may quantitatively or qualitatively affect

10   the activity, either by enhancing or diminishing the activity of the protein in the various assays. Further, individual amino acid residues in the chain may be modified by oxidation, reduction or other derivatization, and the protein may be cleaved to obtain fragments which retain activity. Modification to the primary structure itself by deletion, addition or substitution of the amino acids incorporated into the sequence during

15   translation can be made without destroying the activity of the protein. Such substitutions which do not destroy activity do not remove the protein sequence from the definition and are considered to have substantially equivalent amino acid sequences.

        As used herein the term "transformed" in describing host cell cultures denotes a cell that has been genetically engineered to produce a heterologous protein

20   that possesses the activity of the native protein. Examples of transformed cells are described in the examples of this application. Bacteria are preferred microorganisms for producing the protein. Synthetic protein may also be made by suitable transformed yeast and mammalian host cells.

        The term "refractile material" designates material or bodies which refract

25   light and appear as bright spots in microorganisms when viewed through a phase contrast microscope at magnifications as low as 1000 fold. Refractile material is also known as refractile or inclusion bodies. Examples of heterologous proteins which form refractile bodies in commonly found culture conditions include macrophage colony stimulating factor (M-CSF), interleukin-2 (IL-2), interferon-ß (IFN-ß),

30   envelope protein from feline leukemia virus antigen (FeLV), human growth hormone (hGH), bovine growth hormone (bGH), and certain proteins coated or fused with a virus such as FMD virus. Certain proteins, such as interferon-alpha (IFN-alpha) and tumor necrosis factor (TNF), are more soluble in the cytoplasm.

        "Operably linked" refers to juxtaposition such that the normal function

35   of the components can be performed. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequence can be expressed under the control of these sequences.

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"Control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences which are suitable for procaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet

5 poorly understood, sequences. Eucaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. In order to effect

10 transformation, the expression system may be included on a vector; however, the relevant DNA may then also be integrated into the host chromosome.

As used herein "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom

15 without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny which have the same functionality as screened for in the originally transformed cell, are included. Where indistinct designations are intended, it will be clear from the context.

20 As used herein, the term "pharmaceutically acceptable" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not toxic to the hosts to which it is administered. The administration(s) may take place by any suitable technique, including subcutaneous and parenteral administration, preferably parenteral. Examples of parenteral administration

25 include intravenous, intraarterial, intramuscular, and intraperitoneal, with intravenous being preferred.

As used herein, the term "prophylactic or therapeutic" treatment refers to administration to the host of the multifunctional M-CSF protein either before or after infection or cancer detection. If the multifunctional M-CSF protein is administered

30 prior to exposure to the infecting agent, the treatment is prophylactic (i.e., it protects the host against infection), whereas if administered after infection or initiation of cancer, the treatment is therapeutic (i.e., it combats the existing infection or cancer).

As used herein the term "infectious disease" refers to any kind of infection including those caused by bacteria, fungi, viruses, protozoa or parasites.

35 Examples of sources of bacterial infections include P. aeruginosa, E. coli, tetanus, Mycobacterium species, Streptococcal strains, Corynebacterium diphtheriae and Salmonella. Examples of sources of fungal infection include cryptococcosis,

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histoplasmosis, and other infections due to Candida species. Examples of viral infections include cytomegalovirus (CMV) Hepatitis A, recurrent Herpes Simplex 1 or 2, HIV I or II, Herpes Zoster, influenza, and rhinoviruses.

As used herein, the term "synergistic" refers to the effect of
5 administering the multifunctional proteins disclosed herein being greater than the effect of administering the two or more bioactive components individually. This synergism can allow greater efficacy at an equal dose amount and thereby allow lower dosing resulting in reduced host toxicity.

As used herein, the term "stringent conditions" refers to conditions
10 wherein hybridization is carried out at about 5° below the melting temperature (T_m) of the probe DNA in 5 X SSC (standard saline citrate); 5 X Denhardt's; 50 mM NaPO$_4$, pH 7; 5 mM EDTA; 0.1% SDS; and 200 μg/ml yeast RNA . The effective T_m of the probe DNA can be lowered about 0.7° for every 1% formamide added. While the exact conditions vary with probe length, typical conditions for relatively long probes (e.g.,
15 more than 30-50 nucleotides) employ a temperature of 42°–55°C and the above hybridization buffer containing about 20%-50% formamide. For shorter probes, lower temperatures of about 25°–42°, and lower formamide concentrations (0%-20%) are employed.

The multifunctional proteins of the invention offer an opportunity to link
20 two or more functions in a single molecule which may act simultaneously on target cells. The dual signaling feature of these novel recombinant molecules may result in increased efficacy in the clinic. In addition, larger molecular size has been correlated to increased circulating half-life (Katre, N. et al., 1987, Proc. Natl. Acad. Sci. USA 84:1487-1491). The joining of two or more coding sequences having independent
25 functions or bioactivities has the added advantage of creating a larger molecule with potential for a longer in vivo half-life resulting in increased efficacy.

The M-CSF proteins of the invention are capable both of stimulating monocyte-macrophage cell production from progenitor marrow cells, and of stimulating such functions of these differentiated cells as the secretion of lymphokines in the mature
30 macrophages thus enhancing the effectiveness of the immune system. In general, any subject suffering from immunosuppression whether genetic or due to chemotherapy, bone marrow transplantation, or other, accidental forms of immunosuppression such as disease (e.g., acquired immune deficiency syndrome) would benefit from the availability of M-CSF for pharmacological use. In addition, subjects could be supplied
35 systemically or locally with enhanced amounts of previously differentiated macrophages to supplement those of the indigenous system, which macrophages are produced by in vitro culture of bone marrow or other suitable preparations treated with

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M-CSF. These preparations include those of the patient's own blood monocytes, which can be so cultured and returned for local or systemic therapy. The ability of M-CSF to stimulate production of lymphokines by macrophages and to enhance their ability to kill target cells also makes M-CSF directly useful in treatment of neoplasms
5 and infections.

Human IL-2 has a number of in vitro and in vivo effects including enhancing the proliferative responses of human peripheral blood mononuclear cells or murine thymocytes, enhancing the immune response in humans and in animals against bacterial, parasitic, fungal, protozoan and viral infections, and supporting the growth of
10 continuous T cell lines. rIL-2 has been obtained form genetically engineered E. coli as an unglycosylated protein with biological activities equivalent to those of native, glycosylated IL-2. (Taniguchi et al., 1983, supra; Rosenberg, S.A. et al., 1984, Science 223:1412-1415; Wang, A. et al., 1984, Science 224:1431-1433; and Doyle, M. et al., 1985, J. Biol. Resp. Modifiers 4:96-109). Rosenberg and his coworkers
15 have shown that systemic administration of rIL-2 in high doses causes regression of established metastatic cancers (Rosenberg et al., 1985; J. Exp. Med. 313:1485-1492; Rosenberg et al., 1986, Science 233:1318-1321).

The M-CSF/IL-2 multifunctional protein has synergistic effects compared to the use of either protein separately. This synergism allows greater efficacy
20 at an equal dose amount and thus allows lower dosing resulting in reduced host toxicity. Pharmacokinetics show that the multifunctional protein increases half-life in the plasma when injected in an intravenous bolus compared to the half-life in the plasma of either drug administered separately.

The M-CSF/IL-2 multifunctional protein is tested for in vitro efficacy in
25 cancer therapy and in infectious diseases. Both IL-2 and M-CSF administered individually show a dose-related inhibition of tumor growth in the subcutaneous METH A sarcoma model in BALB/c mice (see Katre, N. et al. supra for IL-2. Both IL-2 and M-CSF proteins also demonstrate activity in the B16 melanoma model against metastases. The multifunctional protein also shows this dose-related inhibition of
30 tumor growth, albeit at substantially lower doses and therefore with substantially reduced toxicity. The beneficial activity of the multifunctional protein is demonstrated in preclinical cancer models, e.g., murine B16 melanoma, in which IL-2 decreases the tumor load therapeutically, and M-CSF decreases the number and size of lung metastases when given prophylactically, for i.v. or s.c. injection of B16 cells as
35 disclosed in PCT Patent Publication No.WO 89/02746 published April 6, 1989 and incorporated herein by reference in its entirety.

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M-CSF and IL-2 are each efficacious when administered independently in various infectious disease models. Bacterial and viral infections may be treated effectively with M-CSF and IL-2 administered individually. For example, M-CSF injections have been shown to protect mice from a lethal E. coli challenge (Chong et al.,
5 1988, FASEB. J. 2(5):A1474) and to protect against fungal Candida infection (Chong, K.T. et al., presented at 1989 American Society for Microbiology Meeting, New Orleans, La.). Cytomegaloviral infection was less severe in M-CSF treated mice. M-CSF has been demonstrated to inhibit vesicular stomatitis virus (VSV) replication and cytopathology in mouse macrophages in vitro (Lee et al., 1987, J. Immunol.
10 138:3019-3022) IL-2 has also been shown to protect mice from a lethal challenge to Gram-negative bacteria such as E. coli and Pseudomonas aeruginosa (European Patent Publication No. 0242233 published October 21, 1987) and to cure infected mice (Gorenzy, J. et al., 1989, J. Immunol 142:1134-1138). IL-2 has been shown to be useful in the treatment of recurrent herpes simplex virus in guinea pigs (Merigan et al.,
15 1988, J. Immunol. 140:294-299). The M-CSF/IL-2 multifunctional protein is also effective in the treatment of bacterial, viral or fungal infections but at a substantially lower dose than either component alone, and therefore with substantially reduced toxicity.

The M-CSF/IL-1 multifunctional protein has synergistic effects
20 compared to the use of either protein separately. M-CSF has been shown to act synergistically with IL-1 in vitro in promoting myeloid cell growth from mouse bone marrow cells (Moore, M.A.S., et al. Recent Advances in Leukemia and Lymphoma, Alan R. Liss, Inc., 445-456; Mochizuki, D.Y. et al., Proc. Natl. Acad. Sci. USA 84:5267-5271; Zsebo, K.M. et al., 1988, Blood 71:962-968). IL-1 has demonstrated
25 synergism with other CSFs such as G-, GM- and multi-CSF or IL-3 for myelopoiesis in vitro (Bartelmez, S.H. et al., Exp. Hematol 17:240-245). In addition, U.S. Patent No. 4,808,611 issued February 28, 1989 incorporated herein by reference in its entirety discloses a method of inducing proliferation and differentiation of hematopoietic stem cells in mammals by the administration of both IL-1 and a CSF.
30 The M-CSF/IL-1 multifunctional protein is more effective than either agent alone, or than the combination of individual agents, at the same dose amounts because the half-lives of each activity in the circulation are increased and the activities are focussed in the bone marrow where they need to be located in order to be effective. For many of these same reasons, the M-CSF/IL-6 multifunctional protein is also useful. (See Bot, F.J. et
35 al., 1989, Blood, 73:435-437)

The M-CSF/IFN-γ multifunctional protein also has synergistic effects compared to the use of either protein separately. Stimulation of mouse macrophages

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(Nakoinz, I. et al., 1988, Cell. Immunol. 116:331-340) and human monocytes (Sampson-Johannes et al., 1988, J. Immunol 114:3580-3686) with the combination of M-CSF and IFN-γ in vitro gave greater cytolytic activity against tumor cells than stimulation by either agent alone. Use of IFN-γ in cancer therapy in vivo has been

5   limited by toxicity even though it is well known as a good stimulator of macrophage anti-tumor activity (Nakoinz et al., supra; Sampson-Johannes et al., supra). A M-CSF/IFN-γ multifunctional protein is ideal for both delivering these macrophage effector cells to the target tumors and for activating their cytolytic capacity. Data has indicated that IFN-γ synergizes with M-CSF when administered concurrently

10   (Nakoinz, I. et al., supra). The multifunctional protein which embodies both activities has a longer half-life in the circulation and serves to direct more of the IFN-γ onto the macrophages, leading to increased efficacy and less toxicity to other cell types.

      Similar efficacy for the M-CSF/IFN-γ multifunctional protein is seen in infectious diseases. M-CSF injections protect mice from lethal in vitro challenge

15   (Chong, et al., supra). M-CSF inhibits vesicular stomatosis virus replication and cytopathology in mouse macrophages in vitro (Lee et al., supra). M-CSF stimulates mouse macrophages to kill intracellular Candida in vitro (Karbassi, A. et al., 1987, J. Immunol. 139:417-421). A number of investigators have shown that IFN-γ stimulates macrophages in vitro to kill or resist infection by a variety of microbial metazoan

20   pathogens. The M-CSF/IFN-γ multifunctional protein is shown to be more efficacious than either protein separately, or separate molecules when mixed together, at the same dose, when tested in these models in vitro or in vivo.

      The M-CSF/G-CSF multifunctional protein provides a means to augment the effect of either CSF-1 and G-CSF administered individually by combining

25   them in therapeutic use. The use of these two factors is synergistic with respect to eliciting enhancement of the immune system. (See Tsuneoka, K. et al., 1984, Cell Structure and Function, 9:67-81 and Metcalf, D. et al., 1985, Leukemia Res., 9:35-50) Together their effect is greater than the sum obtained from simple addition of the effect of either acting alone. It is therefore possible to achieve the desired effect using smaller

30   amounts of the multifunctional protein than would be required by administration of the individual proteins, thus offering the opportunity to reduce side effects and any toxicity which might be associated with elevated doses.

      For parenteral administration the multifunctional proteins of the invention will generally be formulated in a unit dosage injectable form (solution,

35   suspension, emulsion), preferably in a pharmaceutically acceptable carrier medium which is inherently non-toxic and non-therapeutic or non-prophylactic. Examples of

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such vehicles include saline, Ringer's solution, dextrose solution, mannitol, and normal serum albumin.

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well
5 understood in the art (e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982; Ausubel, F.M. et al., eds. Current Protocols in Molecular Biology, Greene Publishing Assoc., New York). Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

10   Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 µg of plasmid or DNA sequence is cleaved by one unit of
15 enzyme in about 20 µl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid
20 recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology, 1980, 65:499-560.

Restriction-cleaved fragments may be blunt ended by treating with the
25 large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 5-10 µM dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTP are present. If desired, selective repair
30 can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

35   Synthetic oligonucleotides may be prepared by the triester method of Matteucci, et al., 1981, J. Am. Chem. Soc. 103:3185-3191 of using automated synthesis methods. Kinasing of single strands prior to annealing or for labeling is

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achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP. If kinasing is for labeling of probe, the ATP will contain high specific activity $\gamma-32P$.

5   Ligations are performed in 15-30 µl volumes under the following standard conditions and temperature: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 µg/ml BSA, 10 mM-50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end"

10 ligations are usually performed at 33-100 µg/ml total DNA concentrations (5-100- nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 µM total ends concentration. Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 µM total ends concentration.

15   In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{+2} using about 1 unit of BAP per µg of vector at 60° for about one hour. In order to

20 recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

        A specific nucleic acid sequence may be cloned into a vector by using

25 primers to amplify the sequence which contain restriction sites on their non-complementary ends according to the general methods as disclosed in U.S. Patent Nos. 4,683,195 issued July 28, 1987, 4,683,202 issued July 28, 1987 and 4,800,159 issued January 24, 1989 the latter of which is incorporated herein by reference in its entirety. A modification of this procedure involving the use of the heat stable Thermus

30 aquaticus (Taq) DNA polymerase has been described and characterized in European Patent Publication No. 258017 published March 2, 1988 incorporated herein by reference in its entirety. Also useful is the Thermal Cycler instrument (Perkin-Elmer-Cetus) which has been described in European Patent Publication No. 236,069, published September 9, 1987 also incorporated herein by reference in its entirety.

35   Generally, the nucleic acid sequence to be cloned is treated with one oligonucleotide primer for each strand and an extension product of each primer is synthesized which is complementary to each nucleic acid strand. An alternative to the

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use of plasmid DNAs encoding the lymphokines of interest as template for PCR is the
use of RNA from any cell producing these lymphokines as template for PCR as
described in U.S. Patent No. 4,800,159. If RNA is the available starting material, the
extension product synthesized from one primer when separated from its complement
5   can serve as template for synthesized of the extension product of the other primer. As
previously mentioned, each primer contains a restriction site on its 5' end which is the
same as or different from the restriction site on the other primer. After sufficient
amplification has occurred the amplification products are treated with the appropriate
restriction enzyme(s) to obtain cleaved products in a restriction digest. The desired
10 fragment to be cloned is then isolated and ligated into the appropriate cloning vector.

For portions of vectors derived from cDNA or genomic DNA which
require sequence modifications, site-specific primer directed mutagenesis is used. This
technique is now standard in the art, and is conducted using a primer synthetic
oligonucleotide complementary to a single stranded phage DNA to be mutagenized
15 except for limited mismatching, representing the desired mutation. Briefly, the
synthetic oligonucleotide is used as a primer to direct synthesis of a strand
complementary to the phage, and the resulting double-stranded DNA is transformed
into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated
in top agar, permitting plaque formation from single cells which harbor the phage.
20   Theoretically, 50% of the new plaques will contain the phage having, as
a single strand, the mutated form; 50% will have the original sequence. The plaques
are transferred to nitrocellulose filters and the "lifts" hybridized with kinased synthetic
primer at a temperature which permits hybridization of an exact match, but at which the
mismatches with the original strand are sufficient to prevent hybridization. Plaques
25 which hybridize with the probe are then picked and cultured, and the DNA is
recovered. Details of site specific mutation procedures are described below in specific
examples.

For example, pCSF-BamBcl contains the entire M-CSF encoding
sequence except that the serine at position 159 is mutated to a stop codon. To
30 accomplish this, the coding sequence was excised from pcCSF-17 and ligated into M13
for site-specific mutagenesis using the primer:

5'- G A G G G A T C C T G A T C A C C G C A G C T C C -3'.
This results in a new BclI site at codons 159-160. The mutated DNA was excised with
BstXI/EcoRI and ligated into the BstXI/EcoRI digested pcCSF-17, the ligation mixture
35 was transformed in E. coli DG105, a dam⁻ host, and the plasmid DNA isolated.

In the constructions set forth below, correct ligations for plasmid
construction are confirmed by first transforming E. coli strain MM294, or other suitable

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host, with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers, depending on the mode of plasmid construction, as is understood in the art. Further screening of transformants is possible using the technique of colony hybridization essentially as

5   described in Maniatis, T. et al. (supra pp. 312-328). Briefly, colonies were lifted onto nitrocellulose filters and sequentially placed on each of four Whatman filters each saturated with one of the following solutions: (1) in 10% SDS; (2) .5MNaOH/1M NaCl; (3) 1.5M NaCl, 1.5M Tris pH 8.0; (4) 2 X SSC for approximately 5 min. each. After cell lysis and binding the DNA, filters were prehybridized for .5-1 hr. at 42°C in

10   hybridization buffer containing 30% formamide followed by hybridization for 1-2 hrs at 42°C. Filters were washed three times in 2 X SSC and 0.1% SDS until background was reduced.

Plasmids from the transformants are then prepared according to the method of Clewell et al., 1969, Proc. Natl. Acad. Sci. (USA) 62:1159, optionally

15   following chloramphenicol amplification (Clewell, 1972, J. Bacteriol 110:667). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy method of Sanger et al., 1977, Proc. Natl. Acad. Sci. (USA) 74:5463 as further described by Messing et al., 1981, Nucleic Acids Res. 9:309, or by the method of Maxam et al., 1980, Methods in Enzymology 65:499.

20   Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N. et al., 1972, Proc. Natl. Acad. Sci. (USA) 69:2110, and modifications as described by Hanahan, D., 1983, J. Mol. Biol., 166:557-580 are used for procaryotes or other cells which contain substantial cell wall

25   barriers. Infection with Agrobacterium tumefaciens (Shaw et al., 1983, Gene 23:315) is used for certain plant cells. Transformations into yeast are carried out according to the method of Van Solingen et al., 1977, J. Bacteriol 130:946 and Hsiao et al., 1979, Proc. Natl. Acad. Sci. (USA) 76:3829.

Several transfection techniques are available for mammalian cells

30   without such cell walls. The calcium phosphate precipitation method of Graham and van der Eb, 1978, Virology 52:546 is one method. Transfection can be carried out using a modification (Wang et al., 1985, Science 228:149) of the calcium phosphate coprecipitation technique. Another transfection technique involves the use of DEAE-dextran (Sompayrac, L.M. et al., 1981, Proc. Natl. Acad. Sci. USA 78:7575-7578).

35   Alternatively, Lipofection refers to a transfection method which uses a lipid matrix to transport plasmid DNA into the host cell. The lipid matrix referred to as Lipofectin™ Reagent is available from BRL. Lipofectin™ Reagent comprises an aqueous solution

(deionized and sterile filtered water) containing 1 mg/ml of lipid (DOTMA:DOPE, 50:50). This liposome-mediated transfection was carried out essentially as described by Feigner, P.L. et al.(1987, Proc. Natl. Acad. Sci. U.S.A. 84:7413). Lipofectin™

5 Reagent and DNA were separately diluted into serum free media so as to avoid gross aggregation which can occur when either material is too concentrated. For example, with 0.5 X 10⁶ cells seeded onto a 60 mm tissue culture dish. 1.5 ml of serum free media containing 1 to 20 µg of DNA and a second solution of 1.5 ml serum free media containing about 30 µg of Lipofectin™ were prepared. The diluted DNA and Lipofectin™ solutions were mixed and applied onto the cells. The transfection is

10 inhibited by serum, so the cells were washed well with serum free media before adding the Lipofectin™/DNA mixture.

The cells were incubated at 37°C for 3 - 24 hours, then 3 ml of media containing 10% serum was added. The incubation time, prior to serum addition required for optimum transfection will vary depending on the cell type and the media

15 used. Transfection with HEPES buffered saline is more cytotoxic and, therefore, shorter incubation times must be used than with EMEM or DMEM; toxicity is somewhat higher when the cells are subconfluent. Particularly good results were obtained with a serum free media, Opti-MEM (GIBCO), using incubation times of up to 24 hours prior to serum addition. The cells were harvested and assayed as usual.

20 For the bone marrow stimulation assay, which measures biological activity of the colony stimulating-factor portion of the protein, bone marrow cells from BALB/c mice were treated with serial dilutions of the culture supernatants, and proliferation of the cells was measured by uptake of labeled thymidine, essentially as described by Moore et al., 1983, J. Immunol. 131:2374 and Prystowsky et al., 1984,

25 Am. J. Pathol. 114:149. Briefly, nucleated bone marrow cells were incubated at 5 X 10⁴ cells well in 96-well plates with dilutions of the samples being tested. After three days [³H]-Thymidine (0.5 µCi/well was added and 6 hours later the cells were harvested and counted. The medium from induced MIA PaCa-2 cells was used as control. Specificity for M-CSF was confirmed by the ability of rabbit antisera raised

30 against human urinary M-CSF to suppress thymidine uptake as disclosed in PCT Patent Publication No. WO 87/04607 published 14 August 1986 incorporated herein by reference in its entirety and in Kawasaki, E.S. et al., supra.

Colony stimulation assays were carried out essentially as described by Metcalf, D., (supra) and Stanley et al., 1972, J. Lab. Clin. Med 79:657 using fetal calf

35 serum (Ralph, P. et al., 1986, supra).

Bone marrow cell suspensions were prepared in bone marrow collecting fluid from the pooled femurs of two to three, two months old C57BL or BALB/c mice.

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Bone marrow collecting fluid consisted of double strength MEM-α medium, 40 ml; fetal calf serum, 10 ml; distilled H_2O, 50 ml.

To prepare the agar culture medium, equal volumes of culture medium and freshly boiled 0.6% Difco Bacto-agar in H_2O were mixed at 37°C and held at

5   37°C. The culture medium was prepared immediately before use by mixing the following: double strength MEM-α medium, 40 ml; fetal calf serum, 10 ml; DEAE dextran (Pharmacia, Sweden) (Dextran MW = 2×10^6 /n/ = 0.70) 50 mgm/ml, 0.15 ml; L-asparagine 6.6 mgm/ml, 0.3 ml.

Sufficient bone marrow cells were added to the agar-medium to give

10   final cell concentrations ranging from 25,000-75,000 cells per milliliter. Cultures were made in 35 mm plastic petri dishes (Falcon Labware, Oxnard, CA) and the source of colony stimulating factor (CSF) was pipetted into the empty petri dishes before the addition of 1 ml of the bone marrow cells in agar-medium. The cells in liquid agar-medium were mixed thoroughly with the source of CSF and the dishes allowed to gel at

15   room temperature. Cultures were incubated for 1-7 days in a fully humidified atmosphere of 10% CO_2 in air.

Counting of cell aggregates were performed on unstained cultures with dissecting microscope at X 25 or 40 magnifications with indirect illumination. Colony size ranged from 50 to 2,000 cells depending on the concentration of CSF. Cytological

20   examination of clusters and colony cells was performed by picking off cell aggregates with a fine Pasteur pipette and placing these on microscope slides. Colonies were stained with 0.6% orcein in 60% acetic acid and cells classified at X 400 or 1000 magnifications according to criteria described previously (Metcalf et al., 1967, J. Natl. Canc. Instit. 39:1235-1245). Alternatively, cell type was determined by

25   cytocentrifuging individual colonies and staining with a modified Wright stain.

Direct assay of the COS-7 supernatants from cells transfected with pcCSF-17 for colony stimulation, for example, showed 4287 U/ml, which was substantially unaffected by the presence of non-immune serum but reduced to 0 U/ml in the presence of rabbit antihuman M-CSF. This compares to 2562 U/ml in the MIA

30   PaCa-2 supernatants. Eighty-five percent of the pcCSF-17 transformed COS-7 supernatant induced colonies had mononuclear/macrophage morphology; MIA PaCa-2 supernatant induced colonies showed a 94% macrophage-6% granulocyte ratio.

The radioreceptor assay measures competition between ^{125}I-labeled M-CSF and the test compound for specific receptors on J774.2 mouse macrophage cells

35   (Ralph, P. et al., 1986, supra; Das, S.K. et al., 1981, supra). MIA PaCa-2 supernatant, assayed for colony stimulating activity as above, was used as a standard (2000 U/ml). The M-CSF concentration of the pcCSF-17 transformed COS-7

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supernatant was found to be 2470 U/ml based on a 1:10 dilution and 3239 U/ml based
on a 1:5 dilution. Thus, comparable values for M-CSF concentration in the media of
COS-7 cells transformed with pcCSF-17 were found in all assays.

An RIA which is specific for human M-CSF has also been described

5 (Shadle, P.J. et al., 1989, Exp. Hematol. 17:154-159 incorporated herein by
reference). Briefly, high titer antisera to human recombinant M-CSF protein allowed
detection of as little as 60 U/ml (1.2 ng/ml) of human M-CSF. The assay consisted of
incubating a mixture of 100 µl of standard, containing 1.2-37.0 ng/ml (60-1910 U/ml)
of M-CSF, 100 µl control or sample, 20,000 cpm of [125I]M-CSF, and 100 µl of

10 diluted rabbit anti-M-CSF for 12-18 h at 4°C. The rabbit antiserum was diluted in
2.5% normal rabbit serum to bind 30%-60% of the added counts of labeled M-CSF
under the assay conditions. An aliquot (50 µl) of goat anti-rabbit IgG, diluted to give
optimal precipitation, was then added, and the mixture was incubated for 1 h at 21°C.
In some assays, the method was modified by adding 0.5 ml of 7.38% polyethylene

15 glycol in phosphate-buffered saline (PBS) with the goat antibody. A 1.5-ml volume of
cold PBS, pH 7.2, was then added, and the bound tracer was separated from free
counts by centrifugation at 2500 g for 20 min. The pellets were then counted for 125I
using a gamma counter. Quantitation of the M-CSF in the samples was determined by
comparison of the sample counts to the counts obtained from the standards.

20 A 48 hour colorimetric assay for M-CSF biological activity has been
developed using M-NFS-60 cell proliferation and MTT (3-(4,5-dimethylthiazol-2-y1)-
2, 5-diphenyl tetrazolium bromide; Sigma) staining in a 96-well tissue culture plate
format. The NFS-60 murine retrovirus-induced myeloid leukemia cell line as isolated
by Weinstein, Y. et al., (1986, Proc. Natl. Acad. Sci. USA 83:5010-5014) was

25 adapted for growth dependence upon M-CSF. Cells were routinely grown in RPMI
1640 medium plus 10% FBS, 1% penicillin/streptomycin, 0.05 mM β-
mercaptoethanol, 2 mM glutamine and 2,000 U/ml recombinant human M-CSF. Cells
for use in the assay were in log phase growth. Cells were washed two times to remove
exogenous M-CSF and diluted to 1 X 10^5 cells/ml in assay medium (growth medium

30 without M-CSF). Wells in rows A to G of microliter plates were filled with 50 µl
assay medium. Samples of unknown activity or standard M-CSF solution (50 µl)
were loaded into column 1 of rows A-G. Serial dilutions (2-fold; 50 µl transfers) were
made from columns 1-12 in rows A-G. Row H contained both blank wells lacking M-
CSF and wells containing a maximally effective amount of M-CSF. Diluted cells were

35 added (50 µl or 5 X 10^3 cells) to each well and plates were incubated 48 hrs at 37°C,
5% CO_2. Then, MTT stain (5 mg/ml) was added (25 µl) to each well. Plates were
incubated at 37°C, 5% CO_2 for 3 hrs. Then 20% (w/v) SDS was added (100 µl/well)

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and plates wrapped tightly in plastic wrap and let stand at room temperature overnight in a light tight box. Well optical densities were read on a BIO-TEK EL310 plate reader at 570 nm.

M-NFS-60 cells proliferate in a dose dependent manner in response to M-CSF or murine IL-3 and also partially respond to G-CSF and murine GM-CSF. M-
5 CSF activity of serially diluted samples are measured relative to a human recombinant M-CSF standard calibrated in a murine bone marrow colony formation assay. Assay results are reported in bone marrow colony forming units/mL (BMCFU /mL). The sensitivity of the M-NFS-60 proliferation assay is approximately 50 BMCFU /mL. The inter-assay precision was determined to be 17% RSD for 47 assays performed over
10 a four month period.

The proliferation of HT-2 mouse helper T-lymphocyte cells in response to IL-2 was measured by a [3H]thymidine ([3H]TdR) incorporation microassay, essentially as described by Gillis et al., 1978, J. Immunol. 120:2027-2032. The target cells were washed and resuspended at 2 X 10^5/ml in RPMI 1640 media containing 10%
15 FBS. Equal volumes of cells and of serial dilutions of IL-2-containing samples were added to 96-well microtiter plates (Falcon/Becton-Dickinson Labware, Oxnard, CA U.S.A.). After 24 h incubation cultures were pulsed for 5 h with 1 μCi [3H]TdR (specific activity, 70 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.), harvested onto Whatman GF/C filters (Whatman Laboratory Products, Inc., Clifton,
20 NJ, U.S.A.), and radioactivity determined in a liquid scintillation counter. IL-2 activity of unknown samples are measured relative to a recombinant human IL-2 standard calibrated in International units.

IL-1 is assayed by commercial Elisas (Cistron, Pine Brook, N.J.; Endogen, Boston, Ma.), by the production of IL-2 by LBRN-33 cells (Larrick, J. et
25 al., 1985, J. Immunol. Meth. 79:39) or by the assays described in U.S. Patent No. 4,808,611 and 4,801,636.

G-CSF, murine GM-CSF and IL-3 are assayed by the NFS-60 assay or the colony stimulation (BMCFU) assay described in Section 6. G-CSF is also measured by assays described in U.S. Patent No. 4,810,643.
30 Murine IFN-γ is assayed for antiviral activity in a cytopathic effect inhibition assay (Stewart, W.E.,II, 1979, The Interferon System, Springer:New York, pp.13-145) with encephalomyocarditis virus as a challenge virus and murine L929 cells as the target. Human IFN-γ is assayed for antiviral activity as described in U.S. Patent No. 4,752,791 in a standard cytopathic effect inhibition assay employing Vesicular
35 Stomatitis Virus (VSV) or Encephalomyocarditis Virus on WISH (human amnion) cells as described by Stewart, supra.

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IL-6 is assayed by a cell line specific assay (Helle, M. et al., 1988, Eur. J. Immunol.18:1535; Shimizu, S. et al., 1989, J. Exp. Med. 169:339).

The expression of plasmid DNAs containing M-CSF fusion genes in COS-A₂ cells was confirmed and quantitated using assays including the bone marrow
5   proliferation assay, the colony stimulation assay, the radioreceptor assay and the M-NFS-60 assay. It will be recalled that the specificity of the bone marrow proliferation assay for M-CSF resides only in the ability of M-CSF antiserum to diminish activity; that for the colony stimulation assay, in the nature of the colonies obtained. The fusion-encoding plasmids were transfected into COS-A2 cells and transient expression of M-
10   CSF activity was assayed by the bone marrow proliferation or the M-NFS 60 proliferation assay and by radioimmunoassay using anti-CSF antibodies.

In general terms, the production of a recombinant fusion form of M-CSF typically involves the following:

First, a DNA is obtained that encodes the fusion protein or preprotein.
15   If the sequence is uninterrupted by introns it is suitable for expression in any host. If there are introns, expression is obtainable in mammalian or other eucaryotic systems capable of processing them. This sequence should be in excisable and recoverable form.

The excised or recovered coding sequence is then preferably placed in
20   operable linkage with suitable control sequences in a replicable expression vector. The vector is used to transform a suitable host and the transformed host cultured under favorable conditions to effect the production of the recombinant M-CSF fusion protein. Optionally the M-CSF fusion protein is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances, where
25   some impurities may be tolerated. However, direct use in therapy by administration to a subject would, of course, require purification of the M-CSF fusion produced.

Each of the foregoing steps can be done in a variety of ways. The constructions for expression vectors operable in a variety of hosts are made using appropriate replicons and control sequences, as set forth below. Suitable restriction
30   sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors.

The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene. Generally, procaryotic, yeast, insert, or mammalian cells are presently useful as host. Since native M-CSF is
35   secreted as a glycosylated dimer, host systems which are capable of proper post-translation processing are preferred. Accordingly, although procaryotic hosts are in general the most efficient and convenient for the production of recombinant proteins,

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eucary...tic cells, and, in particular, mammalian cells or insect cells are preferred for
their processing capacity. Recombinant M-CSF fusion proteins produced by bacteria
may require in vitro dimerization. Such a process for the production of M-CSF
proteins in their active form is disclosed in commonly owned PCT Publication No. WO

5 88/08003 published October 20, 1988 incorporated herein by reference in its entirety.
In addition, there is more assurance that the native signal sequence will be recognized
by mammalian cell or insect cell hosts making secretion possible, and therefore
purification easier.

Procaryotes most frequently are represented by various strains of E.

10 coli. However, other microbial strains may also be used, such as bacilli, for example,
Bacillus subtilis, various species of Pseudomonas, or other bacterial strains. In such
procaryotic systems, plasmid vectors which contain replication sites and control
sequences derived from a species compatible with the host are used. For example, E.
coli is typically transformed using derivatives of pBR322, a plasmid derived from an E.

15 coli species by Bolivar et al., 1977, Gene 2:95. pBR322 contains genes for ampicillin
and tetracycline resistance, and thus provides additional markers which can be either
retained or destroyed in constructing the desired vector. Commonly used procaryotic
control sequences, which are defined herein to include promoters for transcription
initiation, optionally with an operator, along with ribosome binding site sequences,

20 include such commonly used promoters as the beta-lactamase (penicillinase) and lactose
(lac) promoter systems (Chang et al., 1977, Nature 198:1056), the tryptophan (trp)
promoter system (Goeddel et al., 1980, Nucleic Acids Res. 8:4057) and the lambda
derived P_L promoter (Shimatake et al., 1981, Nature 292:128), and N-gene ribosome
binding site, which has been made useful as a portable control cassette, U.S. Patent

25 No. 4,711,845, issued December 8, 1987 and incorporated herein by reference in its
entirety, which comprises a first DNA sequence that is the P_L promoter operably linked
to a second DNA sequence corresponding to the N_{RBS} upstream of a third DNA
sequence having at least one restriction site that permits cleavage within 6 bp 3' of the
N_{RBS} sequence. U.S. Patent No. 4,666,848 issued May 19, 1987 and incorporated

30 herein by reference in its entirety discloses additional vectors with enhanced expression
capabilities. Also useful is the phosphatase A (phoA) system described by Chang et
al., in European Patent Publication No. 196,864, published October 8, 1986,
incorporated herein by reference. However, any available promoter system compatible
with procaryotes can be used.

35 Multifunctional M-CSF fusion proteins are produced in E. coli
preferably using the lambda P_L promoter to direct expression. In a fusion protein

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having M-CSF at the amino-terminal end, the NV_3 form is preferred for expression in
E. coli. The C-terminus may, of course, also be truncated.

     Recombinant M-CSF fusion proteins are recovered from E. coli as
insoluble inclusion bodies or refractile material that are partially purified from the cell
5 debris by centrifugation. SDS-PAGE analysis of the inclusion bodies, following
alkylation or reduction, showed that most of the total M-CSF fusion protein has a
monomeric molecular weight in the absence of reducing agents. This indicates that the
majority of the M-CSF fusion protein in inclusion bodies is non-covalently aggregated.

     The partially purified M-CSF fusion protein in the refractile material is
10 solubilized in urea and purified by size exclusion HPLC (SEC-HPLC) under
denaturing conditions. Following the purification of the reduced M-CSF fusion
monomer, the M-CSF is refolded to its active form by diluting the urea concentration to
less than 1 M. In order to study M-CSF fusion protein refolding reactions at relatively
high protein concentrations, the denatured, monomeric M-CSF fusion protein in the
15 SEC-HPLC pool is concentrated 10-fold to a protein concentration of 5 mg/ml. M-
CSF fusion protein refolding reactions are initiated by diluting the denatured M-CSF
fusion monomer into cold refolding buffer at a final protein concentration of either 0.3
or 0.7 mg/ml. To promote disulfide bond formation and rearrangement, refolding
reactions contain reduced and oxidized glutathione at a ratio of 2:1, respectively. When
20 glutathione (and residual dithiothreitol) are excluded from the refolding reaction, the
rate of M-CSF fusion protein dimerization slowed significantly, requiring five or more
days of refolding to approach completion.

     In addition to bacteria, eucaryotic microbes, such as yeast, may also be
used as hosts. Laboratory strains of Saccharomyces cerevisiae, Baker's yeast, are
25 most used, although a number of other strains are commonly available. While vectors
employing the 2 micron origin of replication are illustrated (Broach, 1983, Meth. Enz.
101:307; U.S. Patent No. 4,803,164 incorporated herein by reference in its entirety),
other plasmid vectors suitable for yeast expression are known (see, for example,
Stinchcomb et al., 1979, Nature 282:39, Tschempe et al., 1980, Gene 10:157 and
30 Clarke et al., 1983, Meth. Enz. 101:300). Control sequences for yeast vectors include
promoters for the synthesis of glycolytic enzymes (Hess et al., 1968, J. Adv. Enzyme.
Reg. 7:149; Holland et al., 1978, Biochemistry 17:4900).

     Additional promoters useful in yeast host microorganisms and known in
the art include the promoter for 3-phosphoglycerate kinase (Hitzeman et al., 1980, J.
35 Biol. Chem. 255: 2073), and those for other glycolytic enzymes, such as
glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase,
phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase,

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pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen
5   metabolism, and enzymes responsible for maltose and galactose utilization (Holland, supra).

It is also believed that terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes. Many of the vectors illustrated
10   contain control sequences derived from the enolase gene containing plasmid peno46 (Holland et al., 1981, J. Biol. Chem. 256:1385) or the LEU2 gene obtained form YEp13 (Broach et al., 1978, Gene 8:121); however, any vector containing a yeast compatible promoter, origin of replication and other control sequences is suitable.

It is also, of course, possible to express genes encoding proteins in
15   eucaryotic host cell cultures derived from multicellular organisms. See, for example, Tissue Culture Academic Press, Cruz and Patterson, editors (1973). Useful host cell lines include murine myelomas N51, VERO and HeLa cells, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the
20   commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers et al., 1978, Nature 273:113) viral promoters such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses, or immunoglobulin promoters and heat shock promoters. A system for expressing DNA in mammalian systems using the BPV as a vector is disclosed in U.S. Patent No. 4,419,446
25   incorporated herein by reference in its entirety. A modification of this system is described in U.S. Patent No. 4,601,978 incorporated herein by reference in its entirety. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Patent No. 4,399,216 issued August 16, 1983. Also useful is gene amplification in eucaryotic cells as described by Ringold in U.S. Patent No. 4,656,134
30   issued April 7, 1987 incorporated herein by reference in its entirety. It now appears also that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream of the promoter region. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes.
35   Plant cells are also now available as hosts, and control sequence compatible with plant cells such as the nopaline synthase promoter and polyadenylation signal sequences (Depicker et al., 1982, J. Mol. Appl. Gen. 1:561) are available.

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Addit:.nally, methods and vectors for transformation of plant cells have been disclosed in PC: Publication No. WO 85/04899 published November 7, 1985 and incorporated herein by reference in its entirety.

Host strains used in cloning and expression herein are as follows:

5   For cloning and sequencing, and for expression of construction under contro: of most bacterial promoters, E. coli strain MM294 obtained from E. coli Genetic Stock Center GCSC #6135, was used as the host. For expression under contro: of the $P_L N_{RBS}$ promoter, E. coli strain K12 MC1000 lambda lysogen, $N_7 N_{5}$:I857 SusP$_{80}$, a strain deposited with the American Type Culture Collection

10   (ATCC 39531), may be used. E. coli DG116, which was deposited with the ATCC (ATCC 53606) on April 7, 1987, may also be used.

For M13 phage recombinants, E. coli strains susceptible to phage infection, such as E. coli K12 strain DG98, are employed. The DG98 strain has been deposited with the ATCC (ATCC 39768) on July 13, 1984.

15   Mammalian expression has been accomplished in COS-A2 cells and also can be accomplished in COS-7, and CV-1, hamster and murine cells. Insect cell-based expression can be in Spodoptera frugiperda.

Examples

The following examples are intended to illustrate but not to limit the

20   invention.

Example I

A. Construction of pML1 Containing SCSF/C ▽158-IL-2 Fusion

The coding sequences encoded by of M-CSF and IL-2 were fused in such a way that the translational reading frame of each of the two proteins was

25   preserved and the resulting fusion protein expressed from this hybrid gene had an amino terminal sequence derived from M-CSF and a carboxy-terminal sequence derived from IL-2. The fusion protein encoded in pML1 contains 158 amino acids of SCSF in addition to the secretory signal sequence at the amino-terminal end and 133 amino acids of IL-2 polypeptide at the carboxy-terminal end. Two additional amino acids were

30   introduced at the fusion junction as a result of the gene construction used.

The plasmids used in the construction of pML1 included pcCSF-17 described in Kawasaki et al., 1985, Supra and deposited on June 14, 1985 having ATCC accession number 53,149. pRAIL102, a ricin A toxin/IL-2 fusion vector, was the source of the IL-2 coding sequence. It contained the following fusion sequence: 5'.

35   . .GT TTT CTT TGC TTA TAA GGC CAA GTG *CTT CCA GGC ACT GGA TCT*

5 3 ⁄ 7

GGC CCG CCG CCT CCG CCG CCT TCT GGA TCC GAG CTT <u>ATG CCT ...</u>-3'
wherein the italicized sequence is the spacer arm and the underlined sequence is the IL-2 coding sequence including a codon for an N-terminal methionine. The sequence 5' to the spacer arm derives from the ricin A and ricin junction peptide coding sequences

5   (European Patent Publication No. 0145111 incorporated herein by reference in its entirety).
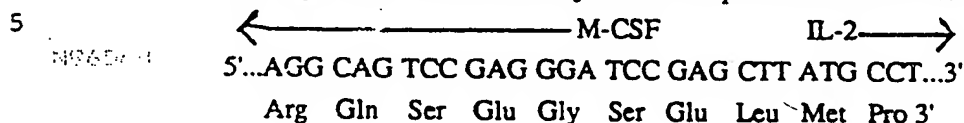
     pRAIL102 was constructed by subcloning the ricin A-spacer arm-IL-2 containing <u>HindIII</u>-<u>Ban</u>II fragment from pRAIL101 into <u>HindIII</u>-<u>Ban</u>II digested pPLOP (deposited December 18, 1984 and having ATCC accession number 39947;

10 also described in U.S. Patent No. 4,677,063 incorporated herein by reference in its entirety). pRAIL101 was constructed by ligating <u>Kpn</u>I digested pPLSA and <u>Hind</u>III digested (blunt ended with Klenow) pLW21 (similar to pLW1 and described in U.S. Patent No. 4,518,584 and in Wang, A. <u>et al</u>., <u>supra</u>). Ampicillin resistant (amp^R) and chloramphenicol resistant (cam^R) transformants were selected and screened for

15 orientation of the des-alanyl IL-2 gene downstream of the ricin A-spacer arm coding sequence. pPLSA contained the ricin A sequence which is encoded in pRA123 (disclosed in copending commonly owned European Patent Publication No. 0,237,676 published September 23, 1987 incorporated herein by reference in its entirety, and deposited August 17, 1984 and having ATCC accession number 39799) fused at the

20 <u>Kpn</u>I site (at codons for amino acids 7-9 of the dodecameric polypeptide linker and therefore preceding the termination codon) to a spacer arm having the sequence:
5'-CTTCCAGGCACTGGATCTGGCCCGCCGCCTCCGCCGCC
TTCTGGATCCGGTACCTGCTGAGTCGAC-3'.

     The <u>BstXI</u>-<u>Bam</u>HI fragment of the <u>BamBcl</u> derivative of pcCSF-17

25 containing the M-CSF secretory leader and mature protein coding sequences through codon 158 as shown in Figure 5 of Kawasaki <u>et al</u>., (<u>supra</u>) was isolated from a 1.2% agarose gel. The <u>Bam</u>HI-<u>Ban</u>II (Klenow repaired) fragment of pRAIL102 containing the des-Ala IL-2$_{cys125}$ gene was also isolated from a 1.2% agarose gel. The vector fragment was from pcDB (Ladner, M.B. <u>et al</u>., 1988, <u>Proc. Natl. Acad. Sci. USA</u>

30 <u>85</u>:6704-6710), a derivative of the Okayama-Berg vector system (Okayama <u>et al</u>., 1982, <u>Mol. Cell. Biol.</u> <u>2</u>:161 and in 1983, <u>Mol. Cell Biol.</u> <u>3</u>:280-289) used in the construction of pcDBCSF-4 (deposited as ATCC accession No. 67250 on October 24, 1986). The <u>Kpn</u>I (Klenow repaired) <u>Bst</u>XI fragment of pcDBCSF-4 (Ladner <u>et al</u>., 1987, <u>supra</u>) was prepared.

35   After ligation of the three fragments, <u>E. coli</u> MM294 was transformed and Ap^R colonies were screened by colony hybridization. Labelled probe EK117 having the sequence 5'-GCTTTGAGCTAAATTTAGCACTTCCTGCAG-3' (codons

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86-95 of the IL-2 gene) was used to identify candidate clones. The candidate designated pML1 was further confirmed to be the desired fusion by XbaI and BstXI double digestion of miniprep DNA.

The sequence across the fusion junction was predicted to be as follows:

$$\longleftarrow \text{————————} \text{M-CSF} \qquad \text{IL-2} \longrightarrow$$

5'...AGG CAG TCC GAG GGA TCC GAG CTT ATG CCT...3'

Arg Gln Ser Glu Gly Ser Glu Leu Met Pro 3'

B. Expression of the Fusion Protein

Monkey COS-A$_2$ cells were transfected with pML1 and DNA containing M-CSF (asp$_{59}$SCSF/CV158; PCT Publication No. WO86/04607) as a control. Cell culture medium was replaced after 16-24 hours and in an additional 48 hours, cell culture medium was harvested, cleared by centrifugation and frozen for further analysis. M-CSF protein in the culture supernatant was assayed by RIA and biological activity was measured in the colony proliferation assay. IL-2 activity was assayed by the HT-2 cell proliferation assay. The results are shown in the Table below.

	M-CSF RIA U/ml	Bone Marrow Colony Assay U/ml	IL-2 Assay U/ml
M-CSF/IL-2 Transfection 1	2.4×10^5	5.7×10^5	4×10^3
M-CSF/IL-2 Transfection 2	2.6×10^5	8.7×10^5	9.2×10^3
pcCSF-17 asp$_{59}$CV158	3.3×10^5	1.9×10^6	0
MOCK	40	0	0

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Figure 1 shows PAGE analysis of transfected COS cell supernatants after
immunoaffinity isolation with rabbit anti-CSF antibody. Lanes 1-3 show unreduced
immunoprecipitates from COS cell supernatants transfected with: lane 1, mock
transfected cells, lane 2, pcCSF17/asp59/ser158/TGA, or lane 3, pML1. Lanes 7-9

5   show reduced immunoprecipitates from Cos cell supernatants transfected with: lane 7,
mock transfected cells, lane 8, pcCSF17/asp59/ser158/TGA, or lane 9, pML1. Lanes 5
and 10 are molecular weight standards as indicated.

Example 2

A. Construction of pML2 Containing LCSF/CV221 - IL-2 Fusion

10   The fusion protein encoded by of pML2 contains 221 amino acids of
LCSF in addition to its secretory signal sequence at the aminoterminal end and 133
amino acids of the mature IL-2 polypeptide at the carboxy-terminal end. The DNA
encoding the fusion protein was derived using the polymerase chain reaction (PCR)
which is described in Section B.2 herein above. The M-CSF coding sequence was

15   amplified from pcDBCSF-4 (deposited October 24, 1986 in the ATCC under accession
number 67250) using primers GM54 and GM55 shown in Table I below. The IL-2
coding sequence was amplified from pML1 (deposited in the ATCC) using primers
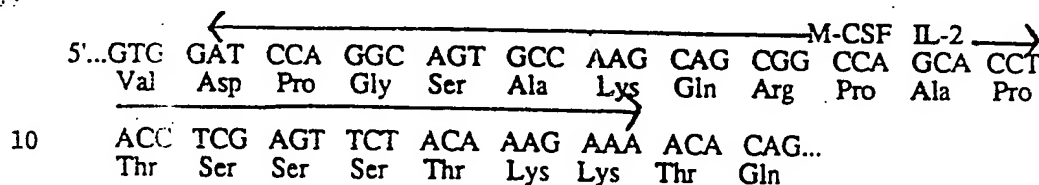GM56 and GM57 also shown below.

Table I of Oligonucleotide Primers

20   GM54 5' GAATTCCATGACCGCGCCGGG 3'
           GM55 5' AACTCGAGGTAGGTGCTGGCCGCTGCTTGGC 3'
           GM56 5' CCTCGAGTTCTACAAAGAAAACACAGC 3'
           GM57 5' GCGGCCGCTTATCAAGTCAGTGTTGAG 3'

GM54 contains an EcoRI recognition site at its 5' end and hybridized to the 5' coding

25   region of M-CSF on the coding strand of pcDBCSF-4. GM55 contains an XhoI
recognition site at its 5' end and hybridized to the 3' end of the M-CSF coding
sequence on the noncoding strand of pcDBCSF-4. PCR amplification resulted in an
EcoRI-XhoI fragment containing the M-CSF coding sequence. Similarly, GM56
contains an XhoI recognition site at its 5' end and hybridized to the 5' end of the IL-2

30   gene on the coding strand of pML1. GM57 contains a NotI recognition site at its 5'
end and hybridized to the 3' end of the IL-2 on the noncoding strand of pML1. PCR
amplification and subsequent restriction enzyme digestion resulted in an XhoI-NotI
fragment containing the IL-2 coding sequence.

35

Ligation of these two PCR-derived fragments into EcoRI and NotI double digested pcDB vector yielded pML2 after transformation into E. coli MM294. Transformants were screened by colony hybridization using GM55 and GM56 as probes and subsequent restriction analysis of miniprep DNAs confirmed the desired
5 fusion construct. DNA sequence analysis of pML2 showed the following sequence spanning the junction of the M-CSF and IL-2 coding sequences.

```
                                                              M-CSF  IL-2
5'...GTG  GAT  CCA  GGC  AGT  GCC  AAG  CAG  CGG  CCA  GCA  CCT
     Val  Asp  Pro  Gly  Ser  Ala  Lys  Gln  Arg  Pro  Ala  Pro

10   ACC  TCG  AGT  TCT  ACA  AAG  AAA  ACA  CAG...
     Thr  Ser  Ser  Ser  Thr  Lys  Lys  Thr  Gln
```

B. Expression of the LCSF/CV221-IL-2 Fusion Protein

Monkey COS-A$_2$ cells were transfected using the lipofection and DEAE-dextran protocols with pML1 and pML2. Cell culture medium was replaced after 16-24
15 hours and in an additional 48 hours, cell culture medium was harvested, cleared by centrifugation and stored frozen. M-CSF protein in the culture supernatant was assayed by RIA and biological activity was measured in the M-NFS-60 assay. IL-2 activity was assayed by the HT-2 cell proliferation assay. The results are shown in the table below.

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	M-CSF RIA U/ml	M-CSF NFS 60 Bioassay U/ml	IL-2 Bioassay U/ml
A. Lipofection Transfection			
pML1 (158-IL-2)	8×10^3	2.07×10^4	3.5×10^3
pML2 (221-IL-2)	1.5×10^3	3.9×10^3	8×10^2
B. Deae - Dextran Transfection			
pML 1	$>3.6 \times 10^4$	1.2×10^5	2.1×10^4
pML 2	1×10^4	2.2×10^4	4.9×10^3
MOCK	<559	<310	<2.24

Example 3

A. Construction of pML3 Containing LCSF/CV221-IL-1α Fusion

The fusion protein of pML3 contains 221 amino acids of LCSF in addition to its secretory signal sequence at the amino-terminal end and amino acids 119 through 271 of human IL-1α. However any bioactive M-CSF or mutein thereof such as SCSF/CV150, SCSF/CV158 and amino-terminal deletions such as NV3 of these or the LCSF molecules are expected to be appropriate for the construction of a dual signaling molecule. Similarly, any amino-terminal deletions of IL-1α may be used. It is known for example that amino acid residues through approximately 127 may be deleted and still retain IL-1α bioactivity (Mosley, B. et al. (1987) Proc. Natl. Acad. Sci. USA 84:4572-4576).

The DNA encoding the fusion protein is derived using PCR as described in Example 2. The M-CSF coding sequence is amplified from pML2 (deposited in the ATCC) using primers GM104 and GM101 shown in Table II below. The resulting 784 bp fragment has a unique BglII site at its 5' end and a unique AsuII site at its 3' end. In addition, the 3' end of this fragment overlaps with the IL-1α coding region for 15 bp. The IL-1α coding region is amplified from pH1-1 (see A.

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Wang) using primer GM102 and GM103 also shown in Table II below. Amplification results in a 484 bp fragment having a 5' AsuII site and a 3' NotI site.

Table II of Oligonucleotide Primers

5
GM 101 5' TTTCACATTCGAAAGTGGCCGCTGCTTGGCACTG 3'
GM 102 5' CAAGCAGCGGCCACTTTCGAATGTGAAATACAAC 3'
GM 103 5' GGGCGGCCGCCTACGCCTGGTTTTCCAGTATC 3'
GM 104 5' CCAGATCTCCATGACCGCGCC 3'

After amplification, DNA fragments were cut with the appropriate restriction enzymes
10 to generate cohesive termini. Following ligation of these fragments into BglII and NotI double digested pcDB vector, E.coli MM294 transformants were selected for ApR. Candidate clones were screened for fusion plasmids containing a single AsuII site, an approximately 1050 bp NotI/BstXI fragment and an approximately 36 bp AsuII/EcoRI fragment. The correct primary sequence across the M-CSF/IL-1α fusion boundary is
15 confirmed by DNA sequence analysis. It is predicted to be:

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$\longleftarrow$————————M-CSF IL-1α————————$\longrightarrow$
5'...GGC AGT GCC AAG CAG CGG CCA CTT TCG AAT GTG AAA TAC AAC...3'
Gly Ser Ala Lys Gln Arg Pro Leu Ser Asn Val Lys Tyr Asn

B. Expression of the LCSF/C—221 - IL-1α

20
Monkey COS-A$_2$ cells are transfected with pML3. Cell culture medium is replaced after 16-24 hours and in an additional 48 hours, cell culture medium is harvested, cleared by centrifugation and stored frozen. M-CSF protein in the culture supernatant is assayed by RIA and biological activity is measured in the M-NFS-60 assay. IL-1α is assayed by the assays described in U.S. Patent Nos. 4,808,611 and
25 4,801,686.

Example 4
M-CSF/γ-IFN Fusion

A. Construction of pML4 Containing LCSF/CV221- γ-IFN Fusion

The fusion protein encoded by pML4 contains 221 amino acids of
30 LCSF in addition to its secretory signal sequence at the amino terminal end and amino acids 1 through 136 of murine γ-IFN. However any bioactive M-CSF or mutein thereof such as SCSF/CV150, SCSF/CV158 and amino-terminal deletions such as NV3 of these or the LCSF molecules are expected to be appropriate for the construction

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of a dual signaling molecule. Similarly, amino-terminal deletions of γ-IFN may be used. Such a deletion for human γ-IFN is disclosed in U.S. Patent No. 4,762,791.

The DNA encoding the fusion protein is derived using PCR as described in Example 2. The M-CSF coding sequence is amplified from pML2 using

5 primers GM104 and MTL02 shown in Table III below. The resulting 800 bp fragment has a unique BglII site at its 5' end and a unique KpnI site at its 3' end. In addition, the 3' end of this fragment overlaps with the IL-1α coding region for 15 bp. The γ-IFN coding region is amplified either from RNA from a γ-IFN producing cell or from a cDNA clone using primer MTL01 and MTL03 also shown in Table III below.

10 Amplification results in a 442 bp fragment having a 5' KpnI site and a 3' NotI site.

Table III of Oligonucleotide Primers

GM 104 5' CCAGATCTCCATGACCGCGCC 3'
MTL01 5' GCAGCGGCCATGTTACTGCCACGGTACCGTCATTGAAAGCC 3'
MTL02 5' GACGGTACCGTGGCAGTAACATGGCCGCTGCTTGGCACTGCC 3'
15 MTL03 5' GGGCGGCCGCCCGAATCAGCAGCGACTCC 3'

After amplification, DNA fragments were cut with the appropriate restriction enzymes to generate cohesive termini. Following ligation of these fragments to BglII and NotI double digested pcDB vector, E.coli MM294 transformants were selected for ApR. Candidate clones were screened for fusion plasmids containing a single KpnI site and

20 an approximately 1184 bp BglII-NotI fragment. The correct primary sequence across the M-CSF/γ-IFN fusion boundary is confirmed by DNA sequence analysis. It is predicted to be:

N96571

$$\longleftarrow \text{————M-CSF IFN-γ ————} \longrightarrow$$

5'...GGC AGT GCC AAG CAG CGG CCA TGT TAC TGC CAC GGT ACC GTC...3'
25   Gly Ser Ala Lys Gln Arg Pro Cys Tyr Cys His Gly Thr Val

B. Expression of the LCSF/CV221 - γIFN

Monkey COS-A2 cells are transfected with pML4. Cell culture medium is replaced after 16-24 hours and in an additional 48 hours, cell culture medium is harvested, cleared by centrifugation and stored frozen. M-CSF protein in the culture

30 supernatant is assayed by RIA and biological activity is measured in the NFS60 assay. γ-IFN is assayed by the assay described in Gray, P.W. et al. (supra).
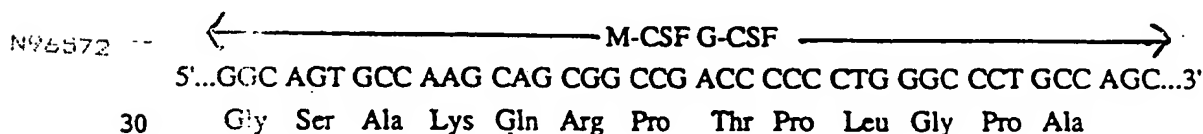
39

Example 5
M-CSF/G-CSF Fusion

A. Construction of pML5 Containing LCSF/CV221 – G-CSF

The fusion protein encoded by pML5 contains 221 amino acids of

5   LCSF in addition to its secretory signal sequence at the amino-terminal end and 174
amino acids of the mature G-CSF polypeptide at the carboxy-terminal end. The DNA
encoding the fusion protein is derived using PCR as described in Example 2. The M-
CSF coding sequence is amplified from pcDBCSF-4 (ATCC No. 67250) using primers
GM 104 and GM 182 shown in Table V below. The resulting 790 bp fragment is

10   digested with BglII which recognizes a site at the 5' end of the fragment and with XhoI
having a site at the 3' end. The G-CSF coding sequence is amplified from pP12
(Devlin, J.J. et al., 1987, J. Leuk. Biol. 41:302-306) using primers GM 183 and GM
184 shown in Table IV. Other sources of the G-CSF sequence include the human
bladder carcinoma cell line 5637 deposited under restrictive conditions as ATCC No.

15   HTB-9 and pJD4A (ATCC No. 6718), pJD4B (ATCC No. 67185) and pPD5A (ATCC
No. 67182). Amplification results in a 558 bp fragment having an XhoI site at the 5'
end and a 3' KpnI site.

Table IV of Oligonucleotide Primers

GM 104 5' CCAGATCTCCATGACCGCGCC 3'

20   GM 182 5'GCTGGCAGGGCCCAGGGGGGTCGGCCGCTGCTTGGCACTGCC3'
GM 183 5'GGCAGTGCCAAGCAGCGGCCGACCCCCCTGGGCCCTGCCAGC3'
GM 184 5'CCATGGTACCTGATCAGGGCTGGGCAAGGTGGCGTAGAAC 3'

After amplification, DNA fragments were cut with the appropriate restriction enzymes
to generate cohesive termini. Following ligation of these fragments into BglII and

25   KpnI double digested pcDB vector, colonies are screened by colony hybridization to
GM182 or GM183. The correct primary sequence across the M-CSF/G-CSF protein
junction is confirmed by DNA sequence analysis. It is predicted to be:

N96572 --   ←——————————————— M-CSF G-CSF ————————————————→

5'...GGC AGT GCC AAG CAG CGG CCG ACC CCC CTG GGC CCT GCC AGC...3'

30   Gly Ser Ala Lys Gln Arg Pro   Thr Pro Leu Gly Pro Ala

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B. Expression of the LCSF/CV221 - G-CSF

  Monkey COS-A$_2$ cells are transfected with pML5. Cell culture medium
is replaced after 16-24 hours and in an additional 48 hours, cell culture medium is
harvested, cleared by centrifugation and stored frozen. M-CSF protein in the culture
5 supernatant is assayed by RIA and biological activity is measured in the M-NFS-60
assay. G-CSF is assayed by bone marrow proliferation and colony stimulation assays
described in Section B.6 and in U.S. Patent No. 4,810,643. Additional G-CSF assays
including immunoassays and cell binding assays are disclosed in U.S. Patent No.
4,810,643.

10   Example 6
         M-CSF/ IL-6 Fusion
A. Construction of pML6 Containing LCSF/CV221 –IL-6

  The fusion protein encoded by pML6 contains 221 amino acids of
LCSF in addition to its secretory signal sequence at the amino-terminal end and 183
15 amino acids of IL-6 at the carboxy-terminal end. The DNA encoding the fusion protein
is amplified from pML2 as described in Example 2. The M-CSF coding sequence is
amplified from pML2 using primers GM104 and GM190 as shown in Table V below.
The resulting 814 bp PCR product is digested with BglII which recognizes a site at the
5' end of the fragment and XmaIII having a site at the 3' end. The IL-6 sequence is
20 amplified either from RNA from an IL-6 producing cell line or from a cDNA clone
using the primers GM187 and GM189 shown in Table V. Amplification results in a
587 bp fragment having a 5' XmaIII site and a 3' KpnI site.
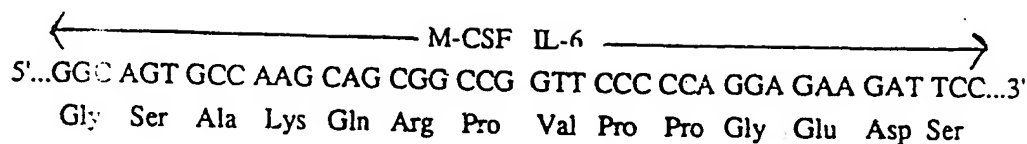
Table V of Oligonucleotide Primers

 GM 104 5' CCAGATCTCCATGACCGCGCC 3'
25 GM 187 5' CCAGGCAGTGCCAAGCAGCGGCCGGTTCCCCCAGGAGAAGATTCC 3'
 GM 190 5' GGAATCTTCTCCTGGGGGAACCGGCCGCTGCTTGGCACTGCCTGG 3'
 GM 189 5' ATGGTACCTTACTACATTTGCCGAAGAGCCCTCAG 3'

After amplification, DNA fragments were cut with the appropriate restriction enzymes
to generate cohesive termini. Following ligation of these fragments into BglII and
30 KpnI digested pcDB vector, colonies are screened by colony hybridization to GM187
or GM 190. The correct primary sequence across the M-CSF/IL-6 junction is
confirmed by DNA sequence analysis. It is predicted to be:

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$$\xleftarrow{\hspace{3cm}}\text{M-CSF}\quad\text{IL-6}\xrightarrow{\hspace{4cm}}$$

5'...GGC AGT GCC AAG CAG CGG CCG GTT CCC CCA GGA GAA GAT TCC...3'

Gly Ser Ala Lys Gln Arg Pro Val Pro Pro Gly Glu Asp Ser

B. Expression of the LCSF/CV221 - IL-6

Monkey COS-A$_2$ cells are transfected with pML6. Cell culture medium is replaced after 16-24 hours and in an additional 48 hours, cell culture medium is harvested, cleared by centrifugation and stored frozen. M-CSF protein in the culture supernatant is assayed by RIA and biological activity is measured in the M-NFS-60 assay. IL-6 is assayed by the method of Helle, M., et al. (supra) or Shimizu, S. et al. (supra).

The following is a list of plasmids which may be useful in practicing the present invention.

Table of Deposits

Plasmid	ATCC No.	Deposit Date	CMCC No.
pcDBCSF-4	67250	October 24, 1986	
pcCSF-17	53149	June 14, 1985	
pcCSF17asp$_{59}$	67139	June 19, 1986	
pcCSF-17gln$_{52}$	67140	June 19, 1986	
pcCSF-17pro$_{52}$	67141	June 19, 1986	
pcCSF-17-Bam	67142	June 19, 1986	
pcCSF-17-BamBcl	67144	June 19, 1986	
pcCSF-17gly$_{152}$	67145	June 19, 1986	
pLW1 (IL-2)	39405		
pLW55 (IL-2)	39516		
pLW45 (IL-2)	39626		
pJD4A (G-CSF)	67181	August 12, 1986	
pJD4B (G-CSF)	67183	August 12, 1986	
pPD5A (G-CSF)	67182	August 12, 1986	
IL-1α	39997		
IL-1β	39925		
pML1		April 18, 1989	3584
pML2		April 18, 1989	3585
pcDB			3583

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These deposits were made for the convenience of the relevant public and do not constitute an admission that a written description would not be sufficient to permit practice of the invention or an intention to limit the invention to these specific constructs. Set forth hereinabove is a complete written description enabling a

5 practitioner of ordinary skill to duplicate the constructs deposited and to construct alternative forms of DNA, or organisms containing it, which permit practice of the invention as claimed.

The scope of the invention is not to be construed as limited by the illustrative embodiments set forth herein, but is to be determined in accordance with the

10 appended claims.

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<u>WHAT IS CLAIMED IS</u>:

1. An isolated DNA sequence which encodes for a multifunctional fusion protein wherein one function of said protein in its active form is to stimulate the formation of primarily macrophage colonies in the <u>in vitro</u> colony stimulating assay of Ralph, P. et al.,1986, <u>Blood 68</u>:633.

2. The DNA sequence of claim 1 wherein said fusion protein is a dimer.

3. The DNA sequence of claim 1 wherein said fusion protein has a second bioactivity associated with a protein selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, α-IFN, β-IFN, γ-IFN, G-CSF, GM-CSF, TNF-α, TNF-β, EPO, thrombopoietin or other platelet enhancing factors, ricin A, diptheria toxin and <u>Pseudomonas</u> exotoxin.

4. The DNA sequence of claim 1 wherein said fusion protein has a second bioactivity associated with a protein selected from the group consisting of IL-1, IL-2, IL-6, γ-IFN and G-CSF.

5. The DNA sequence of claim 4 wherein said second bioactivity is IL-2.

6. The DNA sequence of claim 5 wherein said fusion protein is encoded in a vector selected from the group consisting of pML1 and pML2.

7. The DNA sequence of claim 4 wherein said second bioactivity is IL-1.

8. The DNA sequence of claim 4 wherein said second bioactivity is IL-6.

9. The DNA sequence of claim 4 wherein said second bioactivity is IFN-γ.

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10. The DNA sequence of claim 9 wherein said second bioactivity is human IFN-γ.

11. The DNA sequence of claim 4 wherein said second bioactivity is G-CSF.

12. The DNA sequence of claim 1 wherein said function of stimulating the formation of primarily macrophage colonies in the in vitro assay is encoded 5' to the second bioactivity.

13. The DNA sequence of claim 12 wherein said second bioactivity is IL-2.

14. The DNA sequence of claim 12 wherein said second bioactivity is IL-1.

15. The DNA sequence of claim 12 wherein said second bioactivity is IL-6.

16. The DNA sequence of claim 12 wherein said second bioactivity is IFN-γ.

17. The DNA sequence of claim 16 wherein said second bioactivity is human IFN-γ.

18. The DNA sequence of claim 12 wherein said second bioactivity is G-CSF.

19. An isolated DNA sequence encoding for a multifunctional fusion protein including an amino acid sequence substantially equivalent to the amino acid sequence of M-CSF comprising: Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-Asp-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys-Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu.

20. The DNA sequence of claim 19 which additionally encodes for an amino acid sequence substantially equivalent to the amino acid sequence of IL-2 comprising: Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-Leu-Leu-Asp-Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-Met-Pro-Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Glu-Glu-Leu-Lys-Pro-Leu-Glu-Glu-Val-Leu-Asn-Leu-Ala-Gln-Ser-Lys-Asn-Phe-His-Leu-Arg-Pro-Arg-Asp-Leu-Ile-Ser-Asn-Ile-Asn-Val-Ile-Val-Leu-Glu-Leu-Lys-Gly-Ser-Glu-Thr-Thr-Phe-Met-Cys-Glu-Tyr-Ala-Asp-Glu-Thr-Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser-Ile-Ile-Ser-Thr-Leu-Thr.

21. The DNA sequence of claim 19 which additionally encodes for an amino acid sequence substantially equivalent to the amino acid sequence of IL-1α comprising: Met-Arg-Ile-Ile-Lys-Tyr-Glu-Phe-Ile-Leu-Asn-Asp-Ala-Leu-Asn-Gln-Ser-Ile-Ile-Arg-Ala-Asn-Asp-Gln-Tyr-Leu-Thr-Ala-Ala-Ala-Leu-His-Asn-Leu-Asp-Glu-Ala-Val-Lys-Phe-Asp-Met-Gly-Ala-Tyr-Lys-Ser-Ser-Lys-Asp-Asp-Ala-Lys-Ile-Thr-Val-Ile-Leu-Arg-Ile-Ser-Lys-Thr-Gln-Leu-Tyr-Val-Thr-Ala-Gln-Asp-Glu-Asp-Gln-Pro-Val-Leu-Leu-Lys-Glu-Met-Pro-Glu-Ile-Pro-Lys-Thr-Ile-Thr-Gly-Ser-Glu-Thr-Asn-Leu-Leu-Phe-Phe-Trp-Glu-Thr-His-Gly-Thr-Lys-Asn-Tyr-Phe-Thr-Ser-Val-Ala-His-Pro-Asn-Leu-Phe-Ile-Ala-Thr-Lys-Gln-Asp-Tyr-Trp-Val-Cys-Leu-Ala-Gly-Gly-Pro-Pro-Ser-Ile-Thr-Asp-Phe-Gln-Ile-Leu.

22. The DNA sequence of claim 19 which additionally encodes for an amino acid sequence substantially equivalent to the amino acid sequence of IL-1β comprising: Arg-Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-Ser-Gln-Gln-Lys-Ser-Leu-Val-Met-Ser-Gly-Pro-Tyr-Glu-Leu-Lys-Ala-Leu-His-Leu-Gln-Gly-Gln-Asp-Met-Glu-Gln-Gln-Val-Val-Phe-Ser-Met-Ser-Phe-Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys-Ile-Pro-Val-Ala-Leu-Gly-Leu-Lys-Glu-Lys-Asn-Leu-Tyr-Leu-Ser-Cys-Val-Leu-Lys-Asp-Asp-Lys-Pro-Thr-Leu-Gln-Leu-Glu-Ser-Val-Asp-Pro-Lys-Asn-Tyr-Pro-Lys-Lys-Lys-Met-Glu-Lys-Arg-Phe-Val-Phe-Asn-Lys-Ile-Glu-Ile-Asn-Asn-Lys-Leu-Glu-Phe-Glu-Ser-Ala-Gln-Phe-Pro-Asn-Trp-Tyr-Ile-Ser-Thr-Ser-Gln-Ala-Glu-Asn-Met-Pro-Val-Phe-Leu-Gly-Gly-Thr-Lys-Gly-Gly-Gln-Asp-Ile-Thr-Asp-Phe-Thr-Met-Gln-Phe.

23. The DNA sequence of claim 19 which additionally encodes for an amino acid sequence substantially equivalent to the amino acid sequence of human IFN-γ comprising: Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Ala-Gly-His-Ser-Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-Gly-Ile-Leu-Lys-Asn-Trp-Lys-

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Glu-Glu-Ser-Asp-Arg-Lys-Ile-Met-Gln-Ser-Gln-Ile-Val-Ser-Phe-Tyr-Phe-Lys-Leu-
Phe-Lys-Asn-Phe-Lys-Asp-Asp-Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-
Asp-Met-Asn-Val-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-
Lys-Leu-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-Gln-Arg-Lys-Ala-Ile-His-Glu-
Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-Ser-Pro-Ala-Ala.

24. The DNA sequence of claim 19 which additionally encodes for an
amino acid sequence substantially equivalent to the amino acid sequence of G-CSF
comprising: Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-
Leu-Glu-Gln-Val-Arg-Lys-Ile-Gln-Gly-Asp-Gly-Ala-Ala-Leu-Gln-Glu-Lys-Leu-Cys-
Ala-Thr-Tyr-Lys-Leu-Cys-His-Pro-Glu-Glu-Leu-Val-Leu-Leu-Gly-His-Ser-Leu-Gly-
Ile-Pro-Trp-Ala-Pro-Leu-Ser-Ser-Cys-Pro-Ser-Gln-Ala-Leu-Gln-Leu-Ala-Gly-Cys-
Leu-Ser-Gln-Leu-His-Ser-Gly-Leu-Phe-Leu-Tyr-Gln-Gly-Leu-Leu-Gln-Ala-Leu-Glu-
Gly-Ile-Ser-Pro-Glu-Leu-Gly-Pro-Thr-Leu-Asp-Thr-Leu-Gln-Leu-Asp-Val-Ala-Asp-
Phe-Ala-Thr-Thr-Ile-Trp-Gln-Gln-Met-Glu-Glu-Leu-Gly-Met-Ala-Pro-Ala-Leu-Gln-
Pro-Thr-Gln-Gly-Ala-Met-Pro-Ala-Phe-Ala-Ser-Ala-Phe-Gln-Arg-Arg-Ala-Gly-Gly-
Val-Leu-Val-Ala-Ser-His-Leu-Gln-Ser-Phe-Leu-Glu-Val-Ser-Tyr-Arg-Val-Leu-Arg-
His-Leu-Ala-Gln-Pro.

25. The DNA sequence of claim 19 which additionally encodes for an
amino acid sequence substantially equivalent to the amino acid sequence of IL-6
comprising: Pro-Val-Pro-Pro-Gly-Glu-Asp-Ser-Lys-Asp-Val-Ala-Ala-Pro-His-Arg-
Gln-Pro-Leu-Thr-Ser-Ser-Glu-Arg-Ile-Asp-Lys-Gln-Ile-Arg-Tyr-Ile-Leu-Asp-Gly-Ile-
Ser-Ala-Leu-Arg-Lys-Glu-Thr-Cys-Asn-Lys-Ser-Asn-Met-Cys-Glu-Ser-Ser-Lys-Glu-
Ala-Leu-Ala-Glu-Asn-Asn-Leu-Asn-Leu-Pro-Lys-Met-Ala-Glu-Lys-Asp-Gly-Cys-Phe-
Gln-Ser-Gly-Phe-Asn-Glu-Glu-Thr-Cys-Leu-Val-Lys-Ile-Ile-Thr-Gly-Leu-Leu-Glu-
Phe-Glu-Val-Tyr-Leu-Glu-Try-Leu-Gln-Asn-Arg-Phe-Glu-Ser-Ser-Glu-Glu-Gln-Ala-
Arg-Ala-Val-Gln-Met-Ser-Thr-Lys-Val-Leu-Ile-Gln-Phe-Leu-Gln-Lys-Lys-Ala-Lys-
Asn-Leu-Asp-Ala-Ile-Thr-Thr-Pro-Asp-Pro-Thr-Thr-Asn-Ala-Ser-Leu-Leu-Thr-Lys-
Leu-Gln-Ala-Gln-Asn-Gln-Trp-Leu-Gln-Asp-Met-Thr-Thr-His-Leu-Ile-Leu-Arg-Ser-
Phe-Lys-Glu-Phe-Leu-Gln-Ser-Ser-Leu-Arg-Ala-Leu-Arg-Gln-Met.

26. An isolated DNA sequence comprising coding sequence from a
second protein in addition to M-CSF which hybridizes under stringent conditions to the
the DNA of claim 19 or its complementary strand.

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27. An isolated DNA sequence comprising coding sequence from IL-2 and M-CSF which hybridizes under stringent conditions to the the DNA of claim 20 or its complementary strand.

28. An isolated DNA sequence comprising coding sequence from IL-1α and M-CSF which hybridizes under stringent conditions to the the DNA of claim 21 or its complementary strand.

29. An isolated DNA sequence comprising coding sequence from IL-1β and M-CSF which hybridizes under stringent conditions to the the DNA of claim 22 or its complementary strand.

30. An isolated DNA sequence comprising coding sequence from IFN-γ and M-CSF which hybridizes under stringent conditions to the the DNA of claim 23 or its complementary strand.

31. An isolated DNA sequence comprising coding sequence from G-CSF and M-CSF which hybridizes under stringent conditions to the the DNA of claim 24 or its complementary strand.

32. An isolated DNA sequence comprising coding sequence from IL-6 and M-CSF which hybridizes under stringent conditions to the the DNA of claim 25 or its complementary strand.

33. An isolated DNA sequence comprising coding sequence from a second protein in addition to M-CSF which hybridizes under stringent conditions to the the DNA of claim 1 or its complementary strand.

34. An isolated DNA sequence comprising coding sequence from a second protein in addition to M-CSF which hybridizes under stringent conditions to the the DNA of claim 3 or its complementary strand.

35. An isolated DNA sequence comprising coding sequence from a second protein in addition to M-CSF which hybridizes under stringent conditions to the the DNA of claim 4 or its complementary strand.

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36. A cell culture transformed with a recombinant DNA expression vector capable of expressing the polypeptide encoded by the DNA of claim 1.

37. A cell culture transformed with a recombinant DNA expression vector capable of expressing the polypeptide encoded by the DNA of claim 3.

38. A cell culture transformed with a recombinant DNA expression vector capable of expressing the polypeptide encoded by the DNA of claim 4.

39. A cell culture transformed with a recombinant DNA expression vector capable of expressing the polypeptide encoded by the DNA of claim 5.

40. A cell culture transformed with a recombinant DNA expression vector capable of expressing the polypeptide encoded by the DNA of claim 12.

41. A cell culture transformed with a recombinant DNA expression vector capable of expressing a polypeptide comprising the amino acid sequence of claim 19.

42. A cell culture transformed with a recombinant DNA expression vector capable of expressing a polypeptide comprising the amino acid sequence of claim 20.

43. A cell culture transformed with a recombinant DNA expression vector capable of expressing a polypeptide comprising the amino acid sequence of claim 26.

44. A cell culture transformed with a recombinant DNA expression vector capable of expressing a polypeptide comprising the amino acid sequence of claim 27.

45. An expression system which comprises the DNA of claim 1 operably linked to suitable control sequences.

46. An expression system which comprises the DNA of claim 3 operably linked to suitable control sequences.

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47. An expression system which comprises the DNA of claim 4 operably linked to suitable control sequences.

48. An expression system which comprises the DNA of claim 5 operably linked to suitable control sequences.

49. An expression system which comprises the DNA of claim 12 operably linked to suitable control sequences.

50. An expression system which comprises the DNA of claim 19 operably linked to suitable control sequences.

51. An expression system which comprises the DNA of claim 20 operably linked to suitable control sequences.

52. An expression system which comprises the DNA of claim 26 operably linked to suitable control sequences.

53. An expression system which comprises the DNA of claim 27 operably linked to suitable control sequences.

54. A multifunctional protein wherein one function of said protein in its active form is to stimulate the formation of primarily macrophage colonies in the in vitro colony stimulating assay of Ralph, P. et al., 1986, Blood 68:633.

55. The protein of claim 54 wherein said multifunctional protein is a fusion protein.

56. The protein of claim 55 wherein said multifunctional fusion protein is a dimer.

57. The multifunctional protein of claim 54 comprising a second bioactivity associated with a protein selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, α-IFN, β-IFN, γ-IFN, G-CSF, GM-CSF, TNF-α, TNF-β, EPO, thrombopoietin or other platelet enhancing factors, ricin A, diptheria toxin and Pseudomonas exotoxin.

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58. The multifunctional protein of claim 54 comprising a second bioactivity associated with a protein selected from the group consisting of IL-1, IL-2, IL-6, γ–IFN and G-CSF.

59. The protein of claim 58 wherein said second bioactivity is IL-2.

60. The protein of claim 59 wherein said multifunctional protein is a fusion protein and is encoded in a vector selected from the group consisting of pML1 and pML2.

61. The protein of claim 58 wherein said second bioactivity is IL-1.

62. The protein of claim 58 wherein said second bioactivity is IL-6.

63. The protein of claim 58 wherein said second bioactivity is IFN-γ.

64. The protein of claim 63 wherein said second bioactivity is human IFN-γ.

65. The protein of claim 58 wherein said second bioactivity is G-CSF.

66. The multifunctional protein of claim 55 comprising a second bioactivity associated with a protein selected from the group consisting of IL-1, IL-2, IL-6, γ–IFN and G-CSF wherein said function of stimulating the formation of primarily macrophage colonies in the in vitro assay resides in the amino-terminal end of the molecule.

67. The protein of claim 66 wherein said second bioactivity is IL-2.

68. The protein of claim 66 wherein said second bioactivity is IL-1.

69. The protein of claim 66 wherein said second bioactivity is IL-6.

70. The protein of claim 66 wherein said second bioactivity is IFN-γ.

71. The protein of claim 66 wherein said second bioactivity is human IFN-γ.

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72. The protein of claim 66 wherein said second bioactivity is G-CSF.

73. A multifunctional protein including an amino acid sequence
substantially equivalent to the amino acid sequence of M-CSF comprising: Glu-Tyr-
Cys-Ser-His-Met-Ile-Gly-Ser-Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-
Gln-Met-Glu-Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-Lys-
Asp-Pro-Val-Cys-Tyr-Leu-Lys- Lys-Ala-Phe-Leu-Leu-Val-Gln-Asp-Ile-Met-Glu-Asp-
Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-
Leu-Ser-Leu-Arg-Leu-Lys-Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-
Cys-Val-Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-Asn-Val-
Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-Ile-Phe-Ser-Lys-Asn-Cys-
Asn-Asn-Ser-Phe-Ala-Glu.

74. The protein of claim 73 which is a dimer.

75. The protein of claim 73 which additionally comprises an amino acid
sequence substantially equivalent to the amino acid sequence of IL-2 comprising: Thr-
Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-Leu-Leu-Asp-Leu-Gln-Met-Ile-Leu-Asn-
Gly-Ile-Asn-Asn-Tyr-Lys-Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-
Met-Pro-Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Glu-Glu-Leu-Lys-
Pro-Leu-Glu-Glu-Val-Leu-Asn-Leu-Ala-Gln-Ser-Lys-Asn-Phe-His-Leu-Arg-Pro-Arg-
Asp-Leu-Ile-Ser-Asn-Ile-Asn-Val-Ile-Val-Leu-Glu-Leu-Lys-Gly-Ser-Glu-Thr-Thr-Phe-
Met-Cys-Glu-Tyr-Ala-Asp-Glu-Thr-Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-
Thr-Phe-Cys-Gln-Ser-Ile-Ile-Ser-Thr-Leu-Thr.

76. The protein of claim 73 which additionally comprises an amino acid
sequence substantially equivalent to the amino acid sequence of IL-1α comprising:
Met-Arg-Ile-Ile-Lys-Tyr-Glu-Phe-Ile-Leu-Asn-Asp-Ala-Leu-Asn-Gln-Ser-Ile-Ile-Arg-
Ala-Asn-Asp-Gln-Tyr-Leu-Thr-Ala-Ala-Ala-Leu-His-Asn-Leu-Asp-Glu-Ala-Val-Lys-
Phe-Asp-Met-Gly-Ala-Tyr-Lys-Ser-Ser-Lys-Asp-Asp-Ala-Lys-Ile-Thr-Val-Ile-Leu-
Arg-Ile-Ser-Lys-Thr-Gln-Leu-Tyr-Val-Thr-Ala-Gln-Asp-Glu-Asp-Gln-Pro-Val-Leu-
Leu-Lys-Glu-Met-Pro-Glu-Ile-Pro-Lys-Thr-Ile- Thr-Gly-Ser-Glu-Thr-Asn-Leu-Leu-
Phe-Phe-Trp-Glu-Thr-His-Gly-Thr-Lys-Asn-Tyr-Phe-Thr-Ser-Val-Ala-His-Pro-Asn-
Leu-Phe-Ile-Ala-Thr-Lys-Gln-Asp-Tyr-Trp-Val-Cys-Leu-Ala-Gly-Gly-Pro-Pro-Ser-Ile-
Thr-Asp-Phe-Gln-Ile-Leu.

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77. The protein of claim 73 which additionally comprises an amino acid sequence substantially equivalent to the amino acid sequence of IL-1β comprising: Arg-Ser-Leu-Asn-Cys-Thr-Leu-Arg-Asp-Ser-Gln-Gln-Lys-Ser-Leu-Val-Met-Ser-Gly-Pro-Tyr-Glu-Leu-Lys-Ala-Leu-His-Leu-Gln-Gly-Gln-Asp-Met-Glu-Gln-Gln-Val-Val-Phe-Ser-Met-Ser-Phe-Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys-Ile-Pro-Val-Ala-Leu-Gly-Leu-Lys-Glu-Lys-Asn-Leu-Tyr-Leu-Ser-Cys-Val-Leu-Lys-Asp-Asp-Lys-Pro-Thr-Leu-Gln-Leu-Glu-Ser-Val-Asp-Pro-Lys-Asn-Tyr-Pro-Lys-Lys-Lys-Met-Glu-Lys-Arg-Phe-Val-Phe-Asn-Lys-Ile-Glu-Ile-Asn-Asn-Lys-Leu-Glu-Phe-Glu-Ser-Ala-Gln-Phe-Pro-Asn-Trp-Tyr-Ile-Ser-Thr-Ser-Gln-Ala-Glu-Asn-Met-Pro-Val-Phe-Leu-Gly-Gly-Thr-Lys-Gly-Gly-Gln-Asp-Ile-Thr-Asp-Phe-Thr-Met-Gln-Phe.

78. The protein of claim 73 which additionally comprises an amino acid sequence substantially equivalent to the amino acid sequence of human IFN-γ comprising: Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Ala-Gly-His-Ser-Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-Gly-Ile-Leu-Lys-Asn-Trp-Lys-Glu-Glu-Ser-Asp-Arg-Lys-Ile-Met-Gln-Ser-Gln-Ile-Val-Ser-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-Lys-Asp-Asp-Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-Asp-Met-Asn-Val-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-Lys-Leu-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-Gln-Arg-Lys-Ala-Ile-His-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-Ser-Pro-Ala-Ala.

79. The protein of claim 73 which additionally comprises an amino acid sequence substantially equivalent to the amino acid sequence of G-CSF comprising: Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-Arg-Lys-Ile-Gln-Gly-Asp-Gly-Ala-Ala-Leu-Gln-Glu-Lys-Leu-Cys-Ala-Thr-Tyr-Lys-Leu-Cys-His-Pro-Glu-Glu-Leu-Val-Leu-Leu-Gly-His-Ser-Leu-Gly-Ile-Pro-Trp-Ala-Pro-Leu-Ser-Ser-Cys-Pro-Ser-Gln-Ala-Leu-Gln-Leu-Ala-Gly-Cys-Leu-Ser-Gln-Leu-His-Ser-Gly-Leu-Phe-Leu-Tyr-Gln-Gly-Leu-Leu-Gln-Ala-Leu-Glu-Gly-Ile-Ser-Pro-Glu-Leu-Gly-Pro-Thr-Leu-Asp-Thr-Leu-Gln-Leu-Asp-Val-Ala-Asp-Phe-Ala-Thr-Thr-Ile-Trp-Gln-Gln-Met-Glu-Glu-Leu-Gly-Met-Ala-Pro-Ala-Leu-Gln-Pro-Thr-Gln-Gly-Ala-Met-Pro-Ala-Phe-Ala-Ser-Ala-Phe-Gln-Arg-Arg-Ala-Gly-Gly-Val-Leu-Val-Ala-Ser-His-Leu-Gln-Ser-Phe-Leu-Glu-Val-Ser-Tyr-Arg-Val-Leu-Arg-His-Leu-Ala-Gln-Pro.

80. The protein of claim 73 which additionally comprises an amino acid sequence substantially equivalent to the amino acid sequence of IL-6 comprising: Pro-Val-Pro-Pro-Gly-Glu-Asp-Ser-Lys-Asp-Val-Ala-Ala-Pro-His-Arg-Gln-Pro-Leu-Thr-

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Ser-Ser-Glu-Arg-Ile-Asp-Lys-Gln-Ile-Arg-Tyr-Ile-Leu-Asp-Gly-Ile-Ser-Ala-Leu-Arg-
Lys-Glu-Thr-Cys-Asn-Lys-Ser-Asn-Met-Cys-Glu-Ser-Ser-Lys-Glu-Ala-Leu-Ala-Glu-
Asn-Asn-Leu-Asn-Leu-Pro-Lys-Met-Ala-Glu-Lys-Asp-Gly-Cys-Phe-Gln-Ser-Gly-Phe-
Asn-Glu-Glu-Thr-Cys-Leu-Val-Lys-Ile-Ile-Thr-Gly-Leu-Leu-Glu-Phe-Glu-Val-Tyr-
Leu-Glu-Try-Leu-Gln-Asn-Arg-Phe-Glu-Ser-Ser-Glu-Glu-Gln-Ala-Arg-Ala-Val-Gln-
Met-Ser-Thr-Lys-Val-Leu-Ile-Gln-Phe-Leu-Gln-Lys-Lys-Ala-Lys-Asn-Leu-Asp-Ala-
Ile-Thr-Thr-Pro-Asp-Pro-Thr-Thr-Asn-Ala-Ser-Leu-Leu-Thr-Lys-Leu-Gln-Ala-Gln-
Asn-Gln-Trp-Leu-Gln-Asp-Met-Thr-Thr-His-Leu-Ile-Leu-Arg-Ser-Phe-Lys-Glu-Phe-
Leu-Gln-Ser-Ser-Leu-Arg-Ala-Leu-Arg-Gln-Met.

81. The multifunctional protein of claim 57 wherein said protein is a
heterodimer comprising monomeric subunits each having a different second bioactivity
associated with a protein selected from the group consisting of IL-1, IL-2, IL-3, IL-4,
IL-5, IL-6, IL-7, α-IFN, β-IFN, γ–IFN, G-CSF, GM-CSF, TNF-α, TNF-β, EPO,
thrombopoietin or other platelet enhancing factors, ricin A, diptheria toxin and
Pseudomonas exotoxin.

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